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XXVIII. A GAS ANALYSIS APPARATUS ACCURATE TO 0·001 % MAINLY DESIGNED FOR RESPIRATORY EXCHANGE WORK.

By AUGUST KROGH.

From the Laboratory of Zoophysiology, University of Copenhagen.

(*Received August 26th, 1919.*)

The safest and by far the simplest method of determining the total respiratory exchange of large animals and of man is that introduced by Jaquet, according to which a respiration chamber is ventilated by a current of pure air which is maintained and measured by a gas meter working as a pump. From the current of outgoing air an average sample is collected and analysed, and from the analytical data combined with the ventilation the gas exchange can be calculated. As the determination of the ventilation can easily be made more than accurate enough for the purpose the total accuracy depends on the sampling and the gas analysis.

In gas analysis by current methods [Haldane, 1912; Petterson-Sondén, described by Sondén and Tigerstedt, 1895], the carbon dioxide can be determined with any desired accuracy (to 0·0005 %), but to obtain accurate oxygen determinations is more difficult and even for the best instruments it is not claimed that they can accomplish more than 0·005 % while in actual practice errors of 0·01 % are common enough.

As the oxygen deficit in the air leaving a respiration chamber cannot be increased beyond 2 % and ought not for several reasons to be increased beyond 1 % the accuracy obtainable is scarcely satisfactory, though it must be admitted that, even as it stands, the accuracy of the Jaquet method is certainly not inferior but on the whole superior to that of the Regnault method, the more so as the respiratory quotients obtained with large Regnault apparatus are rather unreliable as shown by the researches of Carpenter [1915].

A great deal would be gained if the accuracy of the gas analysis could be increased sufficiently to allow an oxygen deficit of 0·5–1 % to be analysed without causing errors on the gas exchange exceeding 0·2 %, and this is what I have endeavoured to attain.

The sources of error which prevent the oxygen analyses from being as accurate as the CO_2 determinations are intimately connected with the presence of water and dirt in the gas burette. Water must be present to insure the saturation of the gas with vapour, and dirt will accumulate rather rapidly from the contact of the mercury with the rubber tubing and with oxygen.

In analyses of atmospheric air or the outgoing air from respiration chambers the volume of the burette between the 0 mark at the lower end and the reading after the absorption of oxygen is always about 21 %, and this volume is usually partly expanded into a bulb, as a cylindrical stem with divisions capable of being read to 0·001 % would become inconveniently long. The amount of water and dirt present in this bulb cannot be constant, and this is the chief reason why individual analyses differ. If the burette is kept scrupulously clean and is moistened with water in a constant manner before each analysis, as done by Benedict [1912], the variation of the results can be kept well below ± 0·01 %, and the results will remain comparable over long periods, but when this precaution is not taken the variations become larger, and two analyses of the same air made with an interval during which 5–10 other analyses are carried out may differ considerably. This is due, as pointed out before by A. and M. Krogh [1913], chiefly to the amount of water in the burette which does not remain constant, but decreases steadily by distillation into the absorption pipettes which contain strong solutions of alkali, possessing low vapour tensions[1]. I attempted to obviate the difficulties by measuring the air in a state of complete dryness, but this involved so many difficulties that I had to abandon the plan.

Description of Apparatus.

I have now attained the end desired by two improvements on the usual arrangements.

The most important of these is to use three separate gas burettes (Fig. 1 (1, 2 and 3)) of which one (1) is employed exclusively for moving the air to and from the absorption pipettes, while the second (2) is of a suitable size to measure the air before and after the absorption of CO_2 and the third (3) to measure it after the absorption of O_2. The water vapour necessary for saturating the sample of air, when it has become partially dried in the absorption pipettes, will be supplied by the first burette, and the variations in the amount of water present in this burette will have no influence upon the accuracy of the measurements. The two other burettes (2 and 3) contain just enough water to insure that the sample remains saturated. This water cannot interfere with the readings, because it does not enter the divided stems of the burettes, and its amount will remain constant over a long series of analyses, because the air is always saturated before being brought in contact with it.

The second improvement is that in my apparatus the mercury is raised and lowered in the burettes not by raising and lowering a mercury reservoir but by means of air pressure, an arrangement which obviates the use of rubber connections between the burettes and the reservoirs and moreover facilitates considerably the analytical manipulations.

[1] The soda or potash lye for CO_2 absorption should never be more concentrated than 10 % which is amply sufficient to absorb the CO_2 from an air sample completely and rapidly.

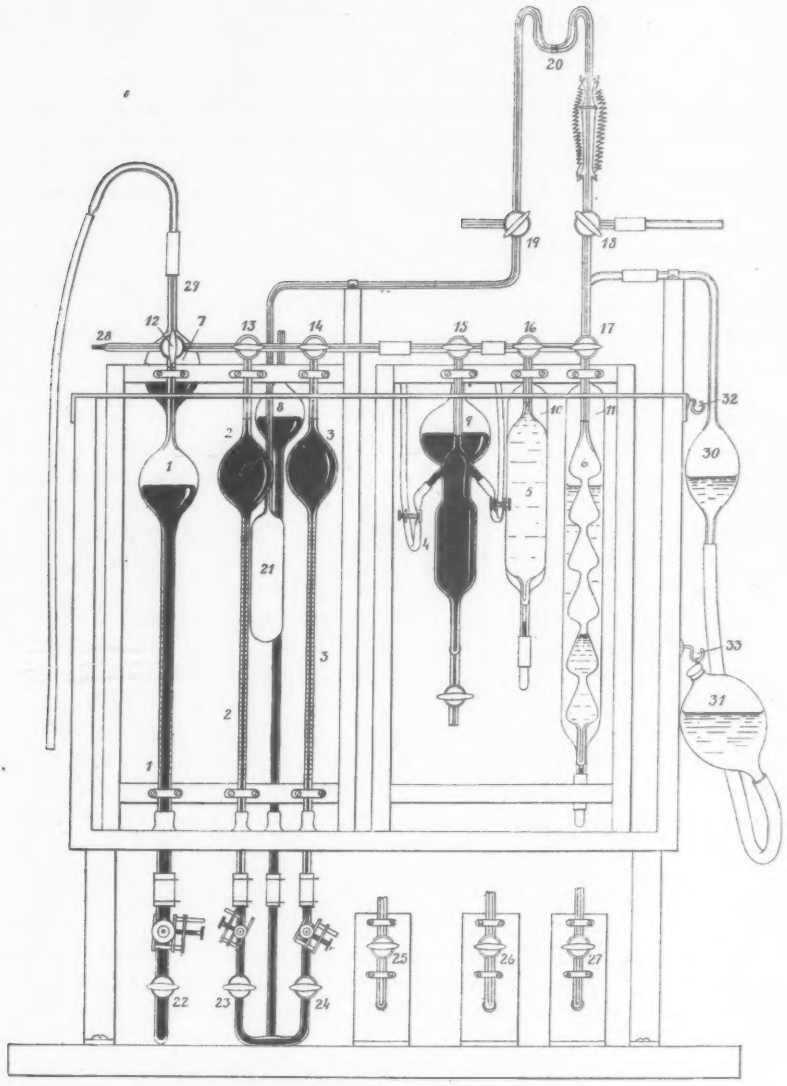
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Fig. 1.

The details of the arrangement for raising and lowering the mercury in one of the burettes is shown in Fig. 2 and parts of it also in Fig. 1.

In Fig. 2 (1) is the burette which is mounted together with the other two and the corresponding reservoirs on a wooden stand (2, comp. Fig. 1). The stems of the burettes and reservoirs are carried through short wide metal tubes (3) in the bottom of the water bath, and a watertight connection which does not expose the glass tubes to any undue strain is brought about by means of soft rubber in the manner shown (4). Below the water bath the burette is connected with the reservoir (9) by means of the U-tube (5) which is provided with the stopcock (6) and the side tube (7). On the side tube is a short piece of stout rubber tubing stoppered with a glass stopper (8). By a screw clip on the rubber a fine adjustment of the level of mercury can be brought about, when the tap (6) is closed. The top of the reservoir (9) is connected by the wide ∩-tube (10) and rubber tubing (11) to the three-way tap (12)[1]. The ∩-tube (10) is interposed to prevent an accidental sucking over of the mercury. The tap (12) is connected through the bottle (13) to a filter pump (14). The same bottle is connected through separate three-way taps with the other reservoirs.

When the tap (12) is open to the atmosphere as shown in Fig. 2 the mercury can rise in the burette to the zero mark at the top, and when by turning tap (12) 90° connection is established with the filter pump, the mercury in the burette can be lowered. The height of the reservoir (9) and the quantity of mercury put into it are so adjusted that the position of equilibrium will bring the meniscus in the burette very near to the zero mark.

Water which is just acidulated by sulphuric acid is introduced into the burettes by taking out the stopcocks (13) and (14) (Fig. 1). In burette 1 about 30 cubic mm. are introduced, and this quantity will last for about 20 analyses (of CO_2 and O_2) corresponding to a loss by distillation of 1·5 cubic mm. during each analysis. In burettes 2 and 3 the quantity of water must be so small that it will always remain in the bulb and not be carried down into the stem, where it would hinder the accurate reading of the mercury meniscus. On the other hand so much water must be present that it is visible as a distinct line along the mercury meniscus when this is anywhere in the bulb. Air is never introduced into any of these burettes directly from the absorption pipettes or from outside but always from burette 1 where it is saturated with moisture.

Burette 2 will in the course of time become a little dirty while 3 will remain clean. The dirt must therefore be caused mainly by oxidation, but we have not been able to detect any diminution in volume when air is left in the burette for a number of minutes.

When a burette has to be cleaned the mercury is lowered to a point just below the side tube (7, Fig. 2) the stopper (8) is removed and a glass tube

[1] The taps (12) are not really placed behind the burettes and reservoirs, but on a line with the taps (6) (see Fig. 1) so as to be easily accessible.

bent at a right angle inserted. Tube (29, Fig. 1) is connected through tap (25) with the filter pump and cleaning fluid (potassium dichromate in dilute sulphuric acid) drawn up. The burette is finally washed with distilled water and dried by a current of air filtered through cotton wool.

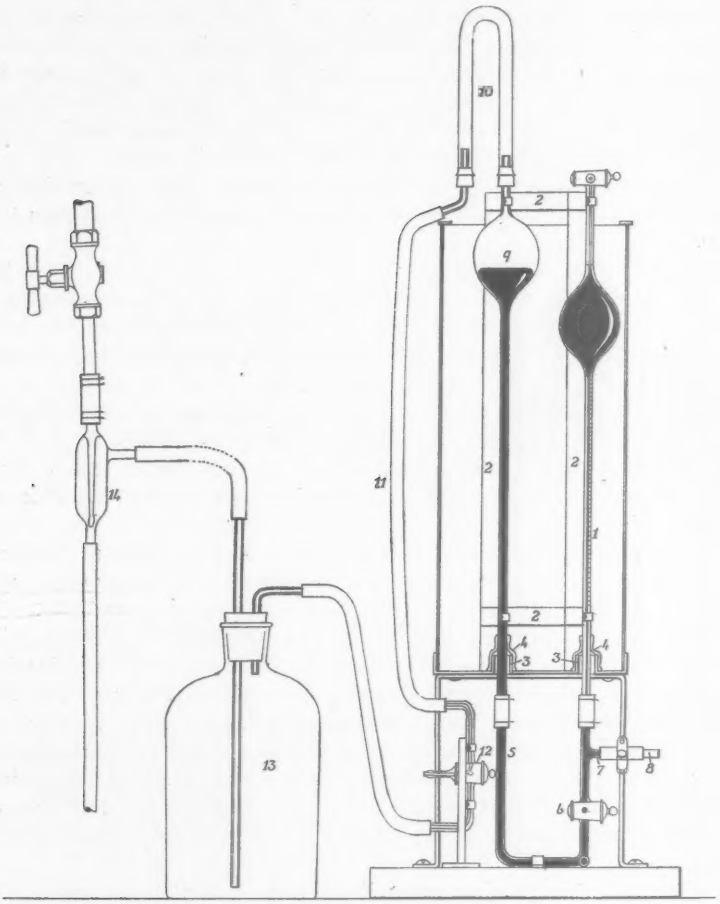

Fig. 2.

The CO_2 absorption pipette (10, Fig. 1), the O_2 absorption pipette (11) and the combustion pipette (9) are mounted together on a wooden stand placed in the same water bath with the burettes. The arrangement of the pipettes has been dictated by several considerations. The main point is to avoid any diffusion of oxygen beyond tap (17) before the absorption takes

place, and this end is fully attained in ordinary analyses in which only CO_2 and O_2 are determined. When, after the absorption of CO_2, connection is established through taps (16, 17 and 18) with the pressure index (20) oxygen will diffuse into the space between taps (16) and (17) but before it can get beyond tap (17) this tap is closed and is not opened again before all (or practically all) the oxygen has been absorbed. If, however, repeated absorptions of CO_2 and corresponding readings become necessary, as in combustion analyses, the possibility cannot be avoided that a trace of oxygen may get beyond tap (17) and escape absorption. Combustion analysis cannot therefore be carried out together with oxygen analysis on the same sample if the greatest possible accuracy of the latter determination is desired.

As the air between tap (17) and the pressure index is always pure nitrogen there is not the slightest danger of contamination of the analysis from this side.

Logically the combustion pipette (4) ought to be placed between the pipettes for CO_2 (5) and for O_2 absorption (6), since combustion is always carried out after the absorption of CO_2. I did not venture however to have the heavy (about 1 kg.) combustion pipette fused on to the other two and therefore preferred to arrange it as shown.

The CO_2 absorption pipette (5) is a plain cylinder with a cylindrical reservoir (10). It is charged with about 10 % potassium hydroxide, carefully saturated with air at the temperature of the bath. It is filled to such a height that the fluid will stand at the mark when the pressures on both sides are equal.

The O_2 absorption pipette (6) is of a peculiar shape designed to insure a maximum of absorption without any danger of small air bubbles being trapped as often happens in pipettes filled with vertical glass tubes. The absorbing fluid for oxygen is Haldane's potash pyrogallate (23 g. of pyrogallic acid of the best quality dissolved in 230 cc. saturated potassium hydroxide, specific gravity 1·55). When air is introduced rapidly into the pipette a considerable quantity of the viscous fluid remains above each contraction and flows slowly downwards, thus providing a large absorbing surface which is automatically renewed so long as the fluid is flowing down. This arrangement shortens considerably the time necessary for complete absorption. One charge of the pipette will last for about 150 analyses before the absorption becomes sluggish.

The construction of the combustion pipette (4, Fig. 1) has been altered after the execution of Fig. 1 and the final shape is shown in Fig. 3. A vertical spiral (1) of fine platinum wire (0·1 mm.) is arranged between two stout wires. Current is supplied through a rheostat from a couple of accumulator cells or from the lighting installation. One pole (2) is connected with the mercury in the pipette and the other (3) through a sealed glass tube with the upper one of the two wires (4). The vertical heated wire, which is placed as far down in the pipette as possible, insures a rapid circulation of the inclosed air,

and the combustion can be completed therefore within a very short space of time—one minute or less.

As the available pressure in the burettes is insufficient to drive an air sample over into the combustion pipette the reservoir (5, Fig. 3) is connected through the three-way tap (27, Fig. 1) with the filter pump.

Method of conducting an Analysis.

An ordinary analysis involving the determination of CO_2 and O_2 is performed as follows (Fig. 1). We suppose all the fluids to be brought exactly to the zero marks on the burettes and pipettes, the three-way taps (13–16) to be turned into their normal position ⊥, the index drop of petroleum (20) to stand at 0, the apparatus to be filled with nitrogen and the pressures in the compensating vessel (21) and the apparatus to be equal. We have the sample of air to be analysed in a glass vessel under a positive pressure. The sampling vessel is connected by means of narrow lead or rubber tubing with the horizontal tube to the left (28). The four-way tap (12) is turned so as to connect the tubes (28) and (29) ⌋, and the connections are washed out with the air to be analysed. The tap (21) is next turned so as to connect the sample with the burette (1) ⅂. The mercury reservoir (7) is connected with the filter pump through the tap (25). The tap (22) on the burette is opened and the sample drawn in. When the mercury reaches the 50 cc. mark on the burette the tap (22) is closed and (25) opened to the atmosphere. A slight positive pressure should now obtain in the sampling vessel and the burette (1). The lead tube is disconnected from the sampling vessel and while the burette (1) is thus open to the atmosphere the excess of pressure blows itself off. The tap (12) is turned so as to connect the burette (1) with the rest of the apparatus ⌜. Tap (13) is turned from the normal position ⊥ into this ⊣, thereby establishing connection between

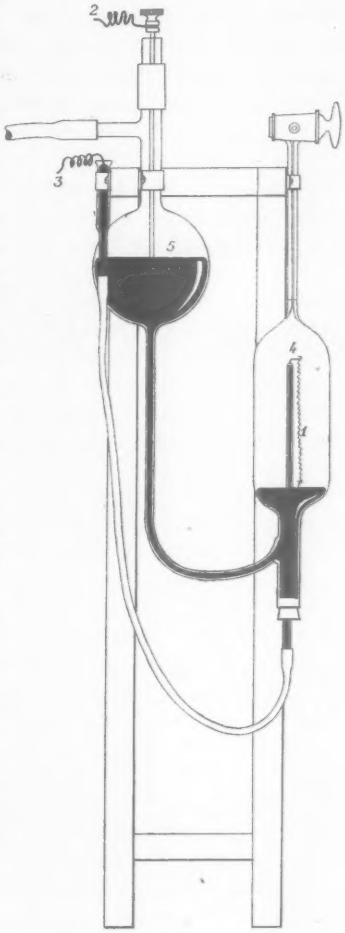

Fig. 3.

the burettes (1) and (2). Taps (22) and (23) are opened, and by opening the tap (26) to the filter pump the sample is transferred to burette (2). When the mercury in (2) has reached the 50 cc. mark on the stem the tap (23) is closed and (26) opened to the atmosphere, and thereupon tap (22) is closed and the water on top of the mercury in burette (1) brought to the zero mark just below tap (12). Tap (13) is turned to the position ⊤ and the taps (18) and (19) connecting the apparatus and the compensating vessel with the pressure index are opened alternately, while the index is watched and the level of the mercury in burette (2) adjusted according to its movements. Finally both taps (19 and 18) are opened (⊢ ⊣), the index set at 0, the burette read and the taps (19 and 18) closed again (⋋ ⋌).

The tap (16) in the potash pipette is now turned from ⊥ to ⊣ and tap (23) opened in consequence of which the sample is driven over into the pipette (5). When the mercury in (2) reaches up to the zero mark tap (23) is closed and usually the mercury is raised by the adjusting screw clip to a little below the tap (13). The tap (22) on burette (1) is now opened and by alternately opening the tap (25) to the filter pump and to the atmosphere the mercury in (1) is lowered and raised 4 times, about 10–15 cc. each time to wash out the connections and insure the complete absorption of the CO_2. This being finished the sample is drawn into burette (1). The potash in (5) is set at the zero mark and the tap (16) closed (≻). In just the same way as before the sample is transferred from (1) to (2), connection is cautiously established through the index with the compensating vessel, the level of mercury in (2) adjusted and read.

Tap (17) is turned so as to establish connection with the O_2 absorption pipette (6) ⅂, and by opening tap (23) the sample is transferred to this pipette. As the available mercury pressure is not sufficient to drive the whole of the sample over into (6) the glass bulb (31) is lowered from the hook (32) to (33). The water in bulbs (30) and (31) protects the pyrogallate solution from contact with the atmosphere. As before the mercury in (2) is brought up to a point a little below the tap (13). By means of burette (1) the sample is taken backwards and forwards to hasten the absorption of oxygen. I have found it most effective to keep it constantly moving. After a suitable number of movements (usually 6) the sample is taken back into (1) and the tap (17) closed (≻). Tap (16) is now opened (⊣) and the sample taken once into the potash pipette (5) in order to "fetch" the air between the zero mark and the tap (16). Thereupon it is again taken into (1). The potash is set at zero and (16) turned to the normal position (⊥). Tap (17) is opened (⅂) and the sample again carried forwards and backwards between burette (1) and pipette (6) to absorb the last traces of oxygen. Finally the sample is taken back into (1), the bulb (31) is raised to (32), the absorbing fluid in (6) set at zero and the tap (17) closed. The water on top of the mercury in burette (2) is now set at zero, the tap (13) turned to the normal position (⊥) and thereupon the sample is transferred from (1) through the tap (14) to burette (3), where it is measured for the third time.

An analysis of CO_2 and O_2 can be made in about 16 minutes. Before the next analysis is made the taps (18) and (19) are opened to the atmosphere (⊤ ⊤) and the nitrogen in burette (3) forced out through (18). The water in (3) is set at zero, the tap (14) turned to the normal position while tap (12) is turned so as to establish connection between tubes (28) and (29) (⌋). When finally taps (19) and (18) are closed (⋌ ⋋) the apparatus is ready for the next analysis.

When a series of analyses is finished the nitrogen is stored in pipette (6), and all taps are closed. Before a new series of analyses is begun all meniscuses are set and finally the nitrogen is driven from burette (1) through the tap (18), which is turned into position ⊣, and the index tube out through the tap (19) (⊥). This is done to insure that the whole system is filled with nitrogen.

A combustion analysis is performed as follows: When the air sample has been measured in burette (2) CO_2 is absorbed and a second reading is taken. Thereupon connection is established between (2) and the combustion pipette (4, Fig. 1). Tap (23) is opened and afterwards tap (27) connecting the reservoir (9) with the filter pump. Between the reservoir and the tap (27) a piece of very narrow tubing is intercalated. Otherwise the movements of the mercury between the pipette (4) and the reservoir (9) would become too violent. When the mercury in pipette (4) has been lowered sufficiently the reservoir is turned off from the pump and the atmosphere and current put on to the platinum wire for one minute. Hydrogen and carbon monoxide will burn readily when the wire is just visibly glowing, but methane will require a bright red heat. A few cc. air can be drawn backwards and forwards between burette (1) and pipette (4), but with the very low percentage of combustible gases which may be present in an ordinary analysis (0–0·01 %) this will scarcely be necessary. After 1 minute's heating the current is shut off and tap (27) opened to the atmosphere. The sample is drawn into burette (1), the meniscus in pipette (4) set, tap (15) closed, the sample transferred to burette (2) and measured. If there is a contraction in volume an absorption of CO_2 is thereupon undertaken.

The volume of air between tap (15) and the zero mark in (5) cannot be subjected to combustion. It is however only about 0·3 % of the total sample. If therefore the percentage of combustible gases does not exceed 0·3 the error committed will be imperceptible. With a higher percentage it can easily be allowed for.

Division of Burettes. Calculation of Analyses.

The stems of burettes 2 and 3 have been selected by myself from a large number of glass tubes and are very nearly cylindrical in bore. Their internal diameter is about 2 mm. and they are divided in ten thousandth parts of 50 cc. The distance between two divisions is approximately 1·6 mm. and it is easy by means of a lens to judge $\frac{1}{10}$ or even $\frac{1}{20}$ of the division. The burettes have been divided and marked with a view to facilitate as much as possible

the calculation of the analyses and especially to make it possible to perform all calculations by means of a simple slide rule.

On burette 2 the point corresponding to 50 cc. has been marked 100, and when the CO_2 percentage in the air analysed is less than 1 (100 divisions) the sample taken should correspond as nearly as possible to this volume. If it is desirable, however, a larger volume can be taken, up to 180. Supposing the first reading of the burette to be $100 + a$ and the second after absorption of the CO_2 to be $100 + b$ we have the CO_2 directly in hundredths % as $a - b$. If the initial reading differs considerably from 100 we must apply a correction which is $-\frac{a(a-b)}{10000}$.

Example. Initial reading 170·0, second reading 20·0. Correction $-\frac{70 \cdot 150}{10000} = 1 \cdot 05$. The corrected figure for CO_2 is therefore $170 - 20 - 1 \cdot 05 = 148 \cdot 95$. When the initial reading is between 90 and 110 and the CO_2 is 50 or less the correction can be omitted altogether, viz. $\frac{10 \cdot 50}{10000} = 0 \cdot 05$. Usually initial readings between 97 and 103 can be secured.

In burette 3 the point corresponding to 79·000 % of 50 cc. is marked 0. If the initial reading is $100 + a$ and the third reading (after absorption of the oxygen) is c we have the nitrogen in hundredths % $= 7900 + c - \frac{79}{100} a$.

Example:

$a =$	2·4
$c =$	11·7
$\frac{79}{100} a =$	1·9
$N_2 =$	9·8 Nitrogen percentage 79·098

If the first reading differs considerably from 100 this figure must be corrected by $-\frac{N_2 \cdot a}{10000}$.

Example:

First reading	175·4	CO_2	$148 \cdot 3 - \frac{148 \cdot 3 \times 75 \cdot 4}{10000} = 147 \cdot 2$
Second reading	27·1		
Third reading	120·3	N_2	$60 \cdot 8 - \frac{60 \cdot 8 \times 75 \cdot 4}{10000} = 60 \cdot 35$
$\frac{79}{100} a$	59·5		

Or in per cent. $CO_2 = 1 \cdot 472$ %, $N_2 = 79 \cdot 6035$ % and O_2 18·9245 %.

In gas exchange work it is never necessary to figure out the actual percentages, because differences only are dealt with.

Example: Analyses of

atmospheric air			outgoing air	
1. 99·4			1. 100·1	
2. 96·05	CO_2 3·35		2. 42·0	CO_2 58·1
3. 3·95	N_2 4·4		3. 12·9	N_2 12·8
$\frac{79}{100} a$ +·45	$CO_2 + N_2$ 7·75		$\frac{79}{100} a$ −·1	$CO_2 + N_2$ 70·9

Differences

CO_2 out. − CO_2 in.	58·1 − 3·35 = 54·75	hundredths of 1 %
N_2 out. − N_2 in.	12·8 − 4·4 = 8·4	,, ,,
O_2 in. − O_2 out.	70·9 − 7·75 = 63·15	,, ,,

The oxygen deficit must be corrected in the usual way, assuming the quantity of nitrogen in the outgoing and ingoing air to be the same. The

correction on the ingoing oxygen (O_i) is $O_i \frac{79{\cdot}00+N_e}{79{\cdot}00+N_i}$. As the fraction $\frac{79{\cdot}00+N_e}{79{\cdot}00+N_i}$ differs very little from unity we can without introducing any perceptible error transform to $\frac{79{\cdot}00+N_e-N_i}{79{\cdot}00}$ or the corrected ingoing oxygen $O_i + \frac{O_i}{79{\cdot}00}(N_e - N_i)$. For the purposes of the correction O_i can be taken as constant and $\frac{O_i}{79} = 0{\cdot}265$. In the example considered the correction on the oxygen deficit is

$$0{\cdot}265 \times 8{\cdot}4 = 2{\cdot}2,$$

the corrected deficit $63{\cdot}15 + 2{\cdot}2 = 65{\cdot}35$ and the respiratory quotient

$$\frac{54{\cdot}75}{65{\cdot}35} = 0{\cdot}838.$$

Accuracy of Relative Analyses for Gas Exchange Work.

In gas exchange work the only practical requirement is that analyses of the same air made within a short space of time (say one day) should agree, but whether the apparatus will give identical results when the same air is analysed repeatedly over a prolonged period is irrelevant. That the apparatus fulfils the conditions of gas exchange work has been thoroughly tested by making regular double determinations of the ingoing atmospheric air and a large number of samples of the outgoing air in actual experiments with a Jaquet respiration chamber. The following series of examples have been selected at random from a very large number and show the accuracy attained in respiratory exchange work when the analyses are performed as rapidly as possible (between 3 and 4 per hour).

Table I. Double determinations.

Date	Atmospheric air			Outgoing air		
	CO_2 %	N_2 %	O_2 deficit %	CO_2 %	N_2 %	O_2 deficit %
	0·0	79·0	0·0	0·	79·	0·
8. v. 1917	335	445	78	5775	1315	709
	335	44	775	5785	1295	708
10. v.	32	425	745	5625	1315	694
	33	395	725	563	1315	6945
11. v.	35	42	77	552	125	677
	33	41	74	553	126	679
14. v.	32	405	725	5785	094	6725
	32	—	—	5775	0935	671
15. v.	315	42	735	600	0775	6775
	33	42	75	600	078	678
17. v.	30	41	71	543	089	632
	305	41	715	544	088	632
18. v.	31	40	71	542	127	669
	32	385	705	541	126	667
21. v.	31	465	775	505	192	697
	315	39	705	506	190	696
22. v.	325	485	81	481	1895	6705
	315	45	765	4805	1895	670
24. v.	35	47	82	499	183	682
	30	46	76	4995	184	6835
25. v.	33	45	78	506	1675	6735
	31	45	76	5055	1685	674
Average difference between double determinations	0·0014	0·0012	0·0025	0·0008	0·0009	0·001

Table II. Double determinations.

Date	Atmospheric air			Outgoing air		
	CO_2 %	N_2 %	O_2 deficit %	CO_2 %	N_2 %	O_2 deficit %
	0·0	79·0	0·0	0·	79·	0·
7. ii. 1918	34	44	78	4655	138	6035
	325	445	77	466	1375	6035
8. ii.	315	51	825	4785	1525	631
	315	525	84	4795	1545	634
9. ii.	305	485	79	4575	149	6065
	31	49	80	456	152	608
10. ii.	30	525	825	4565	1535	610
	31	51	82	457	1545	6115
11. ii.	295	53	825	4685	1235	592
	30	545	845	469	1225	5915
12. ii.	38	57	95	485	102	587
	375	58	955	486	101	587
14. ii.	36	43	79	500	0705	5705
	355	43	785	501	0705	5715
15. ii.	34	455	795	4575	1115	569
	34	465	805	457	1105	5675
17. ii.	39	495	88	4605	158	6185
	395	485	885	460	158	618
	32	44	76	5125	0865	599
	32	445	765	512	087	599
Average difference between double determinations	0·0005	0·0009	0·0009	0·0008	0·0010	0·0010

Table III. Double determinations.

	Atmospheric air						
Date	CO_2 %	N_2 %	O_2 deficit %	Date	CO_2 %	N_2 %	O_2 deficit %
1918	0·0	79·0	0·0	1918	0·0	79·0	0·0
5. iv.	325	555	88	16. iv.	31	485	795
	32	555	875		305	48	785
6. iv.	33	54	87	17. iv.	335	535	87
	325	535	86		33	535	865
7. iv.	32	54	86	19. iv.	30	55	85
	325	555	88		305	545	85
8. iv.	315	48	795	20. iv.	31	525	835
	31	48	79		305	53	835
9. iv.	32	40	72	21. iv.	30	525	825
	32	42	74		295	525	82
10. iv.	32	435	755	22. iv.	305	525	83
	315	43	745		31	515	825
11. iv.	315	465	78	23. iv.	30	525	825
	32	455	775		305	54	845
12. iv.	31	45	76	24. iv.	295	52	815
	315	445	76		30	51	81
13. iv.	315	49	805	25. iv.	315	515	83
	315	48	795		32	51	83
14. iv.	31	455	765	26. iv.	305	515	82
	315	455	77		30	515	815
15. iv.	31	48	79	Average difference	0·0005	0·0006	0·0007
	315	485	80				

The atmospheric air analysed was taken from a Copenhagen street about 20 cm. in front of a window in the laboratory. For the analyses given in Table I two separate samples were taken with an interval of 1–2 hours. Each

sample was taken in less than half a minute, and between the analyses from 2–10 analyses of outgoing air were made. For the analyses in Tables II and III one sample only was taken in six separate portions of about 20 cc. each during the two hour experiment, but one of the analyses was made before and one after a series of 3–6 samples of outgoing air.

The analyses show irregular variations from one day to another both in the CO_2 and in the nitrogen percentages, and these variations are sufficiently large to make it necessary to analyse the ingoing atmospheric air each experimental day, if perfectly reliable results of the gas exchange determinations are to be secured.

The variations observed in the carbon dioxide percentage are real and due to the gas exchange of the town, but the variations in nitrogen are to a large extent of instrumental origin and represent variations in the amount of water present in the measuring burettes.

Further details and a discussion on the composition and possible variations of the atmospheric air have been reported in a separate publication [1920].

Absolute Determinations of Oxygen and Nitrogen.

While in a series of analyses of the same air extended over a prolonged period the CO_2 percentage indicated by the apparatus will remain constant indefinitely, the nitrogen and oxygen may show irregular variations amounting to several thousandths of 1 %. When it is desired to avoid these and to make the determinations absolute, as for instance in investigations of the composition of pure atmospheric air, certain further arrangements and precautions are necessary.

1. The measuring burettes must be very carefully calibrated. The calibration is performed as follows. The burettes are disconnected from the tubing below, cleaned and dried completely by a current of air. A tap is put on to each by means of thick rubber tubing. The junction is made rigid by applying a bandage soaked in gypsum. The lower tube of the tap is drawn out to a point (not too fine) and marked with a line about 5 mm. above the point[1]. A basin containing mercury is arranged below the burette to be calibrated, and by means of burette 1 mercury is drawn up to the desired mark on the stem (care must be taken to avoid air between the tap and the burette). The basin is lowered until the line on the tap is just level with the mercury, the tap is then closed the burette read and the basin weighed. Thereupon the process is repeated, the mercury being drawn up to the zero mark at the top. The difference between the two weighings represents the mercury necessary to fill the burette.

[1] When a second apparatus of this type is built, the lower ends of the measuring burettes will be ground to a standard size, and a tap with a corresponding ground joint provided for calibration purposes.

I reproduce as an example the calibrations made in 1918:

Volumes in cc. at 20°.

Date	T.	Burette 2 0 to 100·0 on stem	Date	T.	Burette 3 0 to 0·0 on stem
13	14·97°	50·0052	13	14·60°	39·4900
14	14·60°	50·0052	13	14·79°	39·4890
14	14·80°	50·0045	14	14·90°	39·4901
	Average	50·0050			39·4897
	Boring of tap 12	0·021			0·021
		50·0260			39·5107

$\frac{7.9}{1.0.6} \times 50{\cdot}0260 = 39{\cdot}5205$

Difference 0·0098 = 0·0195 %

I have found that during the first year after the mounting of the apparatus the volumes of the burettes did not remain constant, but changes amounting to several hundredths of 1 % took place. During the second year and a half the changes observed were very slight, and the volumes have now, probably, become constant.

2. When the dry burettes have been connected with the reservoir and filled with pure and dry mercury a measured quantity of water acidulated with sulphuric acid is introduced into each and preferably 10 cubic mm. into burette 2 and 8 into 3 by which the relation between their volumes is not altered.

3. The water bath must be kept at a constant temperature, lower than that of the room—preferably about 15–16°. To accomplish this it is cooled by means of a current of tap water and heated by a small flame regulated through a sensitive regulator. The lower temperature of the bath will prevent the distillation of water from the burettes to the tubing above.

4. The potassium hydroxide solution must be saturated with air and the pyrogallate solution with nitrogen at ordinary barometric pressure and the temperature of the bath.

5. For absolute determinations the potassium pyrogallate must be tested regarding its absolute absorbing power and the formation of carbon monoxide. The absorbing power can be tested by analysing air with a small proportion of carbon monoxide (1–2 %). When after the absorption of oxygen the residual mixture of nitrogen and CO is put to the test of combustion any residue of oxygen left will cause a contraction of the volume and a formation of carbon dioxide. I have never yet performed this test. I have not the slightest doubt that with a good sample of pyrogallic acid the absorption of oxygen is complete, because the readings become absolutely constant after a very short period of absorption.

When the potassium hydroxide used to make up the pyrogallate solution is not concentrated very appreciable amounts of carbon monoxide are produced by the absorption of oxygen, and I have therefore tried to find out

whether a small quantity of the gas might not possibly be produced also by a concentrated solution. This is done by adding air to the residual nitrogen and subjecting the mixture to combustion analysis. As an average of two series of such determinations I have demonstrated the production of 0·0025 % CO by each analysis. The nitrogen percentage read off must therefore be reduced by subtraction of 0·0025.

6. Even with all the precautions mentioned it is not possible to obtain absolutely constant results over a long time, because a slight accumulation of dirt in burette 2 cannot be avoided, while nothing happens in burette 3 where the mercury is in contact with nitrogen only. Burette 2 must therefore be cleaned frequently and a fresh supply of water introduced after careful drying. The results obtained with a burette which is not quite clean or does not contain the exact quantity of water can be corrected by making control analyses from a stock of standard air alternating with the real analyses. Air which is compressed to 3–5 atmospheres can be stored indefinitely in a strong absolutely dry glass bulb of about 2 litres capacity when it is dried completely before being introduced. I have pumped in the air by means of a bicycle pump through glass tubes with calcium chloride and phosphorus pentoxide. The bulb is arranged as shown in Fig. 4 (1). The reservoir (2) which holds a little over 50 cc. can be connected with the analysis apparatus through the tube (3).

When a control analysis of the standard air shows a deviation which is outside the limits of accidental error (above ± 0·001 % for the nitrogen) the experimental analyses made on the same day should be corrected accordingly.

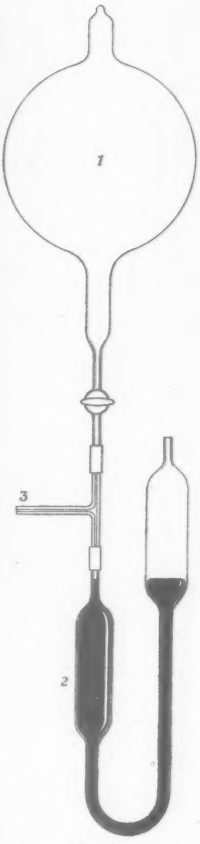

Fig. 4.

Part of the expense of publication of this paper has been defrayed from a grant for which the Biochemical Society is indebted to the Royal Society.

REFERENCES.

Benedict (1912). "The composition of the atmosphere," *Carnegie Publication*, No. 166, Washington.
Carpenter (1915). *Carnegie Publication*, No. 216, Washington.
Haldane (1912). *Methods of air analysis*, London.
Krogh, A. (1920). *Biochem. J.*, **14**.
—— A. and M. (1913). *Medd. om Grönland*, **51**.
Sondén and Tigerstedt (1895). *Skand. Arch. Physiol.*, **6**.

XXIX. THE CALIBRATION, ACCURACY AND USE OF GAS METERS.

By AUGUST KROGH.

From the Laboratory of Zoophysiology, University of Copenhagen.

(*Received August 26th, 1919.*)

Gas meters are widely employed in physiological work for measuring continuous or interrupted currents of air, but they are not always employed correctly and their accuracy is generally underestimated. To this state of affairs it has doubtless contributed that a quantitative examination and calibration, especially of large gas meters, could not be made in the laboratories employing them but had to be left to the manufacturers.

When Professor Lindhard and I installed a Jaquet respiration chamber in the laboratory we were forced by circumstances to put in a meter for producing and measuring the ventilating current of air which was not, in the opinion of the firm, Dansk Maalerfabrik, by whom it had been made, large enough for the purpose, since it had to be used at a maximum rate of 180 revolutions per hour, while wet meters, especially when large, are not considered as accurate beyond 100 revolutions per hour. We had therefore to arrange calibrations at those rates at which we proposed to work it. The apparatus constructed for this purpose has proved very suitable for the examination and calibration of meters generally and some of the results hereby obtained may possibly be of service to other physiologists employing meters.

The Calibration Spirometer.

The calibration spirometer (Fig. 1) is an adaptation of a similar instrument employed by the Dansk Maalerfabrik, the chief improvement being that I have arranged an automatic record of the volume per revolution of the meter calibrated. The drum (1) which is as nearly as possible cylindrical is suspended by a band of soft steel and counterbalanced by the adjustable weight (2). It moves up and down with a minimum of friction. When the drum is in its highest position the weight can be held by an electromagnet (not shown in the figure) from which it can be released at any desired moment by breaking the current. Along the steel band a fine brass wire is arranged carrying a number of lead weights (3). These can be displaced along the wire and a complete equilibrium in all positions of the drum can thus be secured. When the weight (2) is reduced, any desired pressure up to 20 mm. water can be produced in

the spirometer. The revolving fan (4) which can be driven from outside by a motor is used for mixing purposes. In calibration work with air it is revolved for a few minutes when the spirometer has been filled, to secure a uniform temperature and perfect saturation of the air with water vapour.

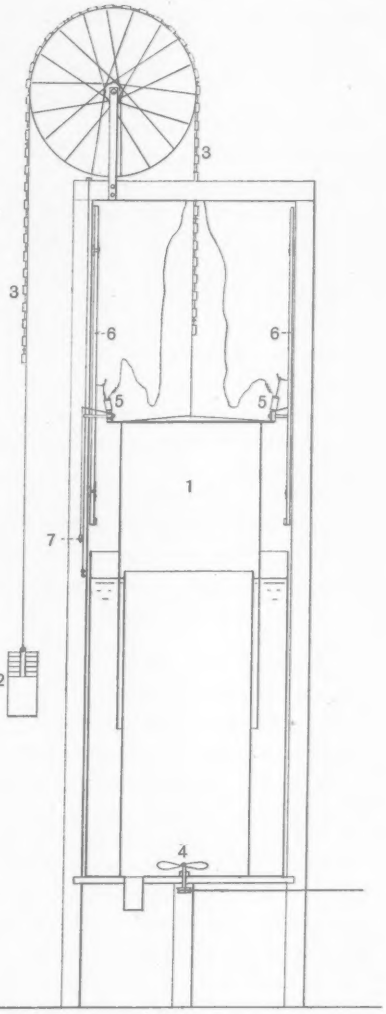

Fig. 1.

The drum is painted inside and outside with a hydrophobe composition so that its volume will not be altered by water adsorbed to its wall. This detail is very important. When left in contact with water, ordinary paint will take up a considerable volume of moisture which cannot drain off. According to experiments a painted tinplate continued to gain in weight while immersed in water during a couple of months, ultimately to the extent of 2·5 g. per 100 sq. cm. Such an increase would in my spirometer diminish by 3·5 cc. the effective volume per cm.

The spirometer drum has been calibrated by measuring a large number of internal diameters (133) evenly distributed over the whole length and circumference of the drum. These measurements have given as the average diameter 450·135 ± 0·09 mm. or a volume of 1591·4 cc. per cm. height. When the drum is used in the spirometer a lowering of 1 cm. will drive out a little more air than the corresponding volume of the drum because the surface of the water in the reservoir will be raised when 1 cm. of the wall of the drum is lowered into it. The volume of 1 cm. of the wall of the drum is 9·4 cc. The rise in the water level will also, of course, correspond to 9·4 cc., but since only 19·0 % of the water surface is inside the drum the extra volume corresponding to 1 cm. will be $\frac{19}{100} \times 9{\cdot}4 = 1{\cdot}8$ cc. and the effective volume of 1 cm. therefore 1593·2 cc. A further correction for the volume of the hydro-

phobe paint and for water adhering to the inside reduces the volume to 1593·0 cc. This latter correction is of course a little arbitrary. When the drum has been rapidly raised the water must be allowed to drain off for about five minutes before a practically constant volume is attained. The water adhering to it at first may amount to a couple of cc. per cm. or more.

The spirometer is arranged to record electrically the revolutions of the meter which is being calibrated. Two electromagnets (5) are placed on the top of the spirometer drum. When the drum is lowered each of them will write a vertical line on a strip of paper stretched out on a detachable board (6). The meter is arranged to close a circuit through the magnets once during each revolution and the magnets will make corresponding notches (7, Fig. 2) on the lines drawn. When the distances between these notches are afterwards measured in cm. the volume per revolution is found by multiplication with 1593·0. As the measurements of diameters have shown that the sectional area of the spirometer drum is not quite uniform all measurements of vertical distances are made from the lowest position of the drum as a base line and a small table has been worked out giving the corrections which must be added to the distances as directly read off. The total working height of the drum is 95 cm. and the largest correction which must be applied between 51 and 68 cm. is − 0·5 mm.

Details of the arrangement of a recording magnet are shown in Fig. 2. The magnet is removed from the strip of paper while the drum is being raised and can be laid on by pulling the thread (8). Eight separate records can be made on a strip of paper 5 cm. broad.

Wet gas meters.

Wet gas meters consist essentially of a measuring drum which can revolve with a minimum of friction in horizontal bearings. The drum is mounted in a box filled with water to a constant level. Meters for commercial purposes are provided with a system of cog-wheels driven by the drum and indicating on dials the gas volumes passing through either in litres or in cubic feet. Meters for scientific purposes (Bohr meters of Dansk Maalerfabrik) have only an electric contact arrangement for counting the revolutions and a subdivision of the volume per revolution generally in 100 parts. The experimental meters are more accurate, partly because they are not influenced by the variable resistance offered by the cog-wheels but also because the volume of one revolution is a constant quantity when the meter is properly handled while the gas volume delivered during a fraction of a revolution is not strictly proportional to the angle through which the drum has been turned. The error may amount in a well-made meter to 1–2 % of the volume of a revolution. In a Bohr meter the subdivisions of the revolution can be calibrated by means of a gas pipette delivering a constant volume, suitably $\frac{1}{10}$ or $\frac{1}{20}$ of the volume of the meter.

Experimental meters are provided with a levelling arrangement which

insures that the axis of the drum shall be horizontal. When they are correctly mounted the volume per revolution depends (1) upon the water level and (2) upon the rate of revolution. Bohr meters have a line marked on the front indicating the proper level of the water. The error which can be committed when the water level is carefully adjusted to this line may according to my experience produce errors of $\pm$ 0·1 % on the volume of the revolution.

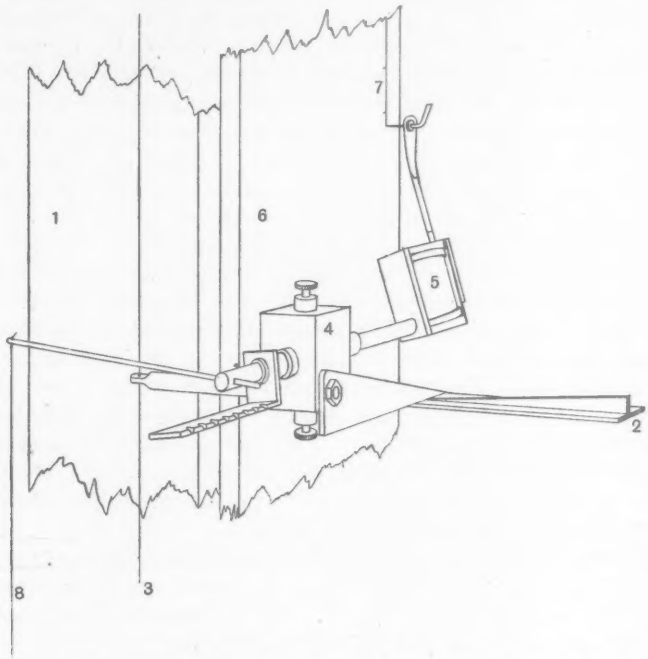

Fig. 2. 1, one of the uprights of the spirometer stand. 2, part of the iron beam across the top of the spirometer drum. 3, steel wire along which the drum is sliding. 4, crosshead with guide for the rod carrying the magnet 5. 6, the detachable board and 7, part of the record with notches corresponding to the revolutions of a meter. 8, the thread for laying on the magnet.

Another arrangement is to have an overflow tube which can be opened and allow any surplus of water to flow off down to the constant level. This arrangement is extremely accurate if sufficient time is allowed to drain off the whole of the excess of water. In experiments with a meter of 14·50 l. per revolution I found that the addition of 100 cc. water, which raised the water level about 1·5 mm., diminished the volume per revolution to 14·40 l. or by 0·7 %. When the surplus of water was allowed to drain off during 12 hours 96 cc. were recovered and the attainable accuracy of the adjustment is therefore of the order $\pm$ 0·03 %.

The volume per revolution of a properly adjusted wet meter is constant within less than 0·1 % at all rates below 1 revolution per minute and for small meters (10 l. per revolution or less) even at all rates below 100 per hour, but when the limit is exceeded there is a definite relation between the rate and the volume. This point has been specially investigated in the case of the 50 candle (4 cb. feet) meter employed for our Jaquet apparatus. This meter is driven by a motor and pumps air out of the respiration chamber. A constant water level is maintained in it irrespective of the rate by a slow current of water (1–2 l. per hour) passing constantly into it and out through the overflow tube. For calibration purposes it is connected by 60 mm. tubing[1] with the spirometer. A water seal which can be rapidly opened and closed is arranged on the tubing at such a distance from the meter that the resistance in the tubing between the seal and the meter corresponds as nearly as possible to the resistance offered by the chamber and tubing in respiration experiments. When the seal is opened the meter draws air from the atmosphere and when it is closed the meter is connected with the spirometer alone. The spirometer drum is loaded so that it will drive out air through the same opening at a rate similar to that at which the meter is pumping. The meter is kept working at a constant rate for a sufficient period to allow the perfect adjustment of the water level, the attainment of which is shown by the inflow and outflow of water being equal. The spirometer drum is raised and held in the highest position, and at a suitable moment shortly before the meter will close the electric circuit the spirometer drum is released and the water seal closed. When the drum approaches the lowest position the water seal is again opened. The meter is so large that only one revolution can be recorded during the sinking of the spirometer drum.

We have made a number of determinations at varying rates of the meter and found that at all rates below 1 revolution per minute the volume of air transported per revolution is constant, while at rates exceeding 1 revolution per minute the volume decreases with increasing rate.

The following numerical results have been obtained:

1 rev. in seconds	142	112	96	94	78	60	45
Vol. per rev. l.	113·98	114·02	114·07	113·96	114·00	114·00	113·55
1 rev. in seconds	33	31	29	24·5	22·5	20·6	18·6
Vol. per rev. l.	113·39	113·20	113·48	113·15	112·78	112·26	112·18

The determinations were made in the course of several days and slow and rapid speeds alternated irregularly. By graphic interpolation a table has been constructed from which the volume per revolution can be taken for all practicable rates of the meter.

If the water level of a meter is adjusted while the meter is at rest and the *quantity* of water remains constant while the rate is changed the volume per

[1] The tubing must be very wide to minimise the resistance which the meter has to overcome. The negative pressure in the drum of the meter should never exceed 5 mm. water. In the present case it is at the most rapid rate used about 2 mm. water.

revolution increases with increasing speed. A 14·5 l. meter was calibrated once by repeated fillings from a 1 l. glass bulb [described and figured by Krogh, 1915] and found to be 14·50 l. The rate in this case was extremely slow. Over a year later the meter was calibrated by means of the spirometer which was loaded so as to drive the air through at a rate of 1 revolution in 39 seconds. The pressure in the spirometer sufficient to produce this rate was 3·6 mm. water. The corrected readings of the record gave

Right mm.	Mean diff. mm.	Left mm.
875·6	—	876·9
784·0	91·55	785·4
692·8	91·2	694·2
601·9	91·0	603·1
510·7	91·15	512·0
419·7	91·15	420·7
328·8	91·0	329·6
237·9	90·8	238·9
147·0	90·9	148·0
56·2	90·9	57·0

The dispersion[1] of the single results is $\delta = 0{\cdot}22$ mm. = 35 cc. and the average is $91{\cdot}07 \pm 0{\cdot}07$ mm. = $14{\cdot}508 \pm 0{\cdot}018$ l. measured in the spirometer at a pressure of 3·6 mm. water above that in the meter. The correction for excess pressure will increase the result by 5 cc. to $14{\cdot}513 \pm 0{\cdot}018$ l. When the rate is increased to 2 revolutions per m. the volume per revolution rises to 14·54 l. and at 3·4 revolutions it is 14·63 l. The corresponding pressures are 7 mm. and 9·5 mm. water.

When a meter is used at a constant rate the constant level arrangement with a current of water will generally be found to be the most suitable as no errors can arise from evaporation, but when the rate is inconstant or even discontinuous the constant quantity is the only possibility.

In respiration experiments, when a meter is used to measure the air expired from the lungs of man or an animal, the maximum rate during expiration can be taken to be approximately three times the total rate and care should be taken that the fastest rate does not exceed the capacity of the meter.

Dry gas meters.

Dry gas meters consist of two bellows which are alternately filled and emptied by the current of air passing through. They require no filling and will work equally well in any position. As the fractions of one revolution are often very incorrectly shown by a dry meter they should never for experimental purposes be divided in litres as commercial dry meters are but simply have a device for counting the revolutions.

[1] The term "dispersion" has been introduced by Charlier to take the place of "standard deviation."

A dry 10 candle meter (volume per revolution about 8 l.) arranged with one dial measuring 10 l. and divided in tenths of a litre was tested by passing air through it from the one litre bulb. The successive readings gave

Reading l.	7·0	7·6	8·4	9·35	0·25	1·5	2·75	3·75	4·8	5·55	6·25	7·25
Diff. = 1 l.		0·6	0·8	0·95	0·9	1·25	1·25	1·0	1·05	0·75	0·7	1·0

The readings corresponding to 1 l. vary in this case between 0·6 and 1·25 l. but repeated tests starting from the same point gave very different results. When the meter was tested by the calibration spirometer it was found that successive revolutions of the hand indicating 10 l. per revolution showed similar variations, but since the volume of the drum was a submultiple of the volume corresponding to 50 l. the results were repeated with astounding regularity for each 50 l. The true volumes corresponding to successive readings of 10 l. were

0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90	90–100
10·32	9·85	9·83	9·99	10·62	10·32	9·85	9·83	9·99	10·62

The volume corresponding to a reading of 100 l. is 101·2.

A 5 candle meter arranged to count the true revolutions and subdivided into $\frac{1}{100}$ parts of a revolution gave similar though smaller variations when tested by means of the 1 l. glass bulb but in this case a repeated test gave approximately the same errors at corresponding divisions. When tested by means of the calibration spirometer the volume per revolution proved constant so long as the rate remained constant.

The indications of a dry meter depend generally to a certain extent upon the rate, but the effect of the rate may differ in different meters. For the meters mentioned above I have found

10 candle meter		5 candle meter		
100 l. in	100·9 l.	1 rev. in 45 seconds	L. per revolution 5·10	Pressure mm. water 3
300 seconds	101·1	22	5·08	6
150	101·2	13	5·04	15
145	101·8			
110				

The volume per revolution of a dry meter will not remain unchanged indefinitely, because the material from which the bellows are made will deteriorate. Within a year the change appears to be very slight but this of course depends upon the initial quality and age of the material. After nine months the calibration of the new 10 candle meter mentioned above has changed from 100·9 to 101·3 l. per 100 l. indicated.

Summary.

A large recording spirometer for calibrating gas meters is described.

Wet gas meters properly handled are instruments of precision. Motor-driven wet meters acting as pumps and with a constant water level maintained by a slow current of water are accurate to less than 0·1 % at all rates below

1 revolution per minute. When they are calibrated for more rapid rates they can be used with almost the same accuracy at rates up to 3 revolutions per minute. The volume per revolution decreases with increasing rate.

In wet meters with a constant quantity of water the volume per revolution increases with increasing rate but can be determined with equal accuracy.

The subdivisions of the revolution of an experimental wet meter (Bohr meter) usually show slight errors which can be allowed for after calibration.

Dry gas meters are on the whole less accurate than wet, and when they are arranged to show the volumes directly in litres their indications are often very inaccurate and cannot be calibrated. They should be arranged to count revolutions.

Gas volumes representing fractions of a complete revolution are generally very inaccurately indicated but the volume corresponding to a whole revolution is a practically constant quantity. Varying rates may cause variations in the volume registered but at practicable rates usually within 1 %. The calibration of a dry meter changes with age.

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REFERENCE.

Krogh (1915). *Abderhalden's Handbuch Biochem. Arbeitsmet.*, **8**, 537.

XXX. THE RELATIVE VALUE OF FAT AND CARBOHYDRATE AS SOURCES OF MUSCULAR ENERGY.

WITH APPENDICES ON THE CORRELATION BETWEEN STANDARD METABOLISM AND THE RESPIRATORY QUOTIENT DURING REST AND WORK.

By AUGUST KROGH and JOHANNES LINDHARD,

WITH THE COLLABORATION OF

GÖRAN LILJESTRAND and KNUD GAD ANDRESEN.

From the Laboratory of Zoophysiology, Copenhagen University.

(*Received August 26th, 1919.*)

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Introduction.

A number of researches on isolated muscles, made most of them during the last decade by the Cambridge School of physiologists, have revived the old problem about the immediate source of muscular energy, in so far as their main results cannot be reconciled to the commonly accepted view, that the

muscle is able to make use indiscriminately and with the same coefficient of utilisation of different sources of energy. According to this view, which is based mainly on the researches of Zuntz and his school, the muscular machine can transform a definite fraction of the energy liberated by the combustion of any available substance into mechanical work—provided the substance can be oxidised in the organism. According to the Cambridge school the muscular contraction itself does not depend at all upon an oxidative reaction but upon the splitting up of a definite unknown molecule, resulting in the formation of lactic acid, while other definite processes, involving the recombination of lactic acid into the molecule directly concerned in the contraction, are necessary to restore the contractility.

The researches on which the supposed isodynamic value for muscular work of very different oxidisable substances are based have, without exception, been made on the organism as a whole, and the general principle has been to feed a subject during a certain period upon a diet consisting chiefly of a single foodstuff (fat, protein or carbohydrate), to let the subject perform a definite amount of work and to study the relation between the work performed and the corresponding metabolism. When a number of such periods, which differed only in the character of the chief substance catabolised, were compared the conclusion was arrived at by Zuntz [1911] and his collaborators that the coefficient of utilisation is practically independent of the substance catabolised[1].

From the researches of Fletcher, Hopkins, A. V. Hill and their collaborators [see Fletcher and Hopkins, 1917] it must be inferred on the other hand that, when the muscular machine requires certain reactions to take place between definite substances which must be closely allied to carbohydrates, it is almost inconceivable that substances such as fats can be utilised without a transformation involving loss of energy, and one is led to expect therefore that the coefficient of utilisation should be lower for other substances than for carbohydrates.

We do not propose to enter upon a discussion of the Cambridge results, but the discrepancy between them and those of Zuntz has caused a growing feeling of uneasiness in our minds which has at last compelled us to take up the problem and to see whether Zuntz's conclusion will stand the test of a renewed experimental investigation.

The general plan of our proposed research was briefly as follows. A human subject should live for a certain number of days on a definite diet, containing a minimum of protein and a very decided preponderance of either fat or carbohydrate. During this period, or part of it, he should come to the laboratory in the morning before taking any food and with as little muscular effort as

[1] It should be remembered, however, that the researches made in Zuntz's laboratory were not primarily intended to demonstrate the equal value of the different foodstuffs, but to examine whether the 30 % difference in value between fat and carbohydrate postulated by the hypothesis of Chauveau could be found or not.

possible. After a suitable period of complete rest, during which the standard metabolism could be measured, he should work on a bicycle ergometer for a certain length of time, which we fixed provisionally at two hours, and the rate at which work was performed should be kept constant throughout for the same subject. During the work a number of determinations of the respiratory exchange should be made. After a suitable number of days the diet should be altered and the determinations repeated.

The experimental difficulties involved in such an investigation are very considerable, and it was evident from the first that a definite solution of our problem could only be hoped for when the accuracy of all the determinations could be raised to a high standard. The reliability must depend (1) on the technical accuracy of the determinations themselves, (2) on the constancy of the external conditions to which the subject was exposed and (3) on the constancy of reaction on the part of the subject. The greatest difficulty is presented by the subject. During a total experimental period of three weeks or a month effects of training and probably other changes are likely to occur, and it was impossible to tell beforehand how these could best be avoided or their influence minimised. We arranged therefore to make several series of experiments and to use the results of the first to guide us in the planning of the following. It is evident that a condition for doing this is that we should be able to recognise which variations in the results must be due to the variations in the reaction of the subject and which to errors in the determinations, and it was imperative therefore that the technical accuracy of the determinations should be as great as possible.

We had to choose between determining the pulmonary gas exchange by measurements and analyses of the expired air and determining the total gas exchange in a chamber. Although the methods for measuring the pulmonary gas exchange can give very accurate results and have been used in almost all former researches bearing upon our problem we thought it better to discard them because they would involve that the subject should wear a respiration mask (or a mouthpiece) during a period of two hours, which would become rather disagreeable and might influence the metabolism in unaccountable ways[1].

When the determinations should be made by means of a respiration chamber we had to choose between the closed circuit (Regnault) and the air current (Jaquet) type of apparatus. The dangers and drawbacks of large Regnault apparatus have been pointed out by Krogh [1915, 2] and from the interesting control experiments reported by Carpenter [1915] it is clear,

[1] In the excellent series of experiments on muscular work on an ergometer made by Benedict and Cathcart [1913] the subject was connected with the mouthpiece for a number of short periods only which were distributed over the time of riding the ergometer. This device appears to us to involve serious dangers. For a certain time after the putting on of a mouthpiece and noseclip the respiration is apt to be distinctly abnormal and though the absorption of oxygen is as a rule scarcely affected by such changes the CO_2 output may become abnormal by washing out, when the ventilation is increased, and storing up when it is diminished.

moreover, that the accurate determination of respiratory quotients in a closed circuit apparatus is—to say the least—very difficult to attain.

Carpenter has carried out extensive comparisons between respiration apparatus of various types. The comparisons were made between instruments of two types at a time by employing them for alternate determinations of the respiratory exchange of the same subject. Usually about six alternate determinations were made on each experimental day, and care was taken to change the order in which the instruments were used from day to day. This method of experimenting is extremely suitable to bring out both the uniformity of results (accidental variations) obtained with each type and its absolute reliability (systematic error). The accidental variations have been given by Carpenter in "probability curves" but the systematic differences between the types have not been discussed. It is obvious, however, that when the averages of long series of such determinations differ by more than double the mean error of each average the difference cannot be purely accidental, but must be due to some inherent peculiarity in one or both of the apparatus.

From the probability curves given by Carpenter it can be deduced that the mean error on the average of each separate series of determinations of the respiratory quotient is of the order $\pm 0{\cdot}005$ and nearly the same for the different types of apparatus except the "Spirometer Unit" and the "Bed Calorimeter" which have slightly larger errors.

Table I.

Apparatus	Number of exp.	CO_2 cc./min.	O_2 cc./min.	R.Q.	R.Q. reduced
1	2	3	4	5	6
T.E. Unit	142	185	227	0·815	
Bed Cal.	95	190	223	0·85	0·865
T.E. Unit	31	197	231	0·855	
Sp. Unit	25	198	233	$0{\cdot}85 \pm {\cdot}005$	0·83
T.E. Unit	36	190	224	0·85	
Zuntz-Gep.	35	186	227	0·82	0·80
Sp. Unit	74	182	219	0·83	
Zuntz-Gep.	58	176	220	$0{\cdot}80 \pm {\cdot}005$	0·80
T.E. Unit	44	165	193	0·855	
Tissot	37	167	194	0·86	0·835
Sp. Unit	65	190	233	0·815	
Tissot	52	192	242	0·795	0·81
Sp. Unit	48	189	231	0·82	
Douglas	45	178	224	0·795	0·805

In Table I we have put together the average results of the various comparisons, compiled from Carpenter's book. The figures in column 5 show that while the "Tension Equaliser Unit" and the "Spirometer Unit" give the same average quotients, the differences found in the other comparisons are too large to be accidental.

In column 6 we have arranged a direct comparison between all the types of apparatus investigated by reducing the respiratory quotients found in the various series by means of the same apparatus (the "Spirometer Unit") to a uniform value put arbitrarily at 0·83 and it is seen that the "Bed Calorimeter," which is a large closed circuit apparatus, gives a much higher average quotient, while the Zuntz-Geppert and Douglas apparatus, in which the quotient depends exclusively on the results of a gas analysis, give only 0·80. (In the comparisons with the Tissot apparatus very special errors come into play which it is not deemed necessary to discuss.)

The respiratory quotient determined by gas analysis of the expired (and inspired) air correctly performed cannot have any *systematic* error, and it follows that the respiratory quotients determined by means of the closed circuit "Unit" apparatus are on an average 0·03 too high, while those found by means of the respiration calorimeter are 0·065 too high.

In a Jaquet apparatus on the other hand the accuracy of the respiratory quotient depends exclusively on the gas analysis. There is therefore no danger of systematic errors in the quotient while the limits of the accidental errors can always be controlled. A special gas analysis apparatus capable of analysing the respiratory gases to 0·001 % has been constructed for this and similar researches and described by Krogh [1920, 1].

Description of Apparatus and Method.

Our Jaquet apparatus which has been constructed on the lines laid down by Grafe [1910] is shown in Fig. 1. The chamber consists of a framework of angle iron to which sheets of galvanised iron have been riveted and made airtight by soldering. The form and dimensions have been chosen with a view to accommodating the bicycle ergometer and the subject riding the machine. The floor is made from a single sheet of galvanised iron with the edges bent downwards into a U shaped rectangular groove (1, Fig. 1) which is filled with water. The chamber itself dips into the same water seal when closed during an experiment. As shown in the figure (at 2) one end can be lifted to let in the subject and put in apparatus. At the other end just above the groove where the movements are very slight a number of small tubes (3) are arranged to introduce wires etc. for the working of the ergometer, mixing fans, signals etc.

Near the top of the chamber are two wider tubes (4 and 5). Through a mouthpiece or mask, respiration valves and flexible tubing the subject can be connected with one of these (4) and through it with a dry meter placed outside the chamber. The air for inspiration has in such experiments been taken from the chamber and again returned to the chamber from the meter through the tube (5). This arrangement has been used during some introductory experiments to compare the pulmonary gas exchange with the total.

The chamber is ventilated with outside air from the street through a 60 mm. tube (6) which is connected up at the beginning of an experiment. At the other end of the chamber, a similar tube (7), which is likewise put in place when the chamber has been let down, connects it with the gas meter. The inlet tube simply opens into the chamber, but the outlet tube draws air simultaneously from three points distant about 50 cm. from each other (8).

During experiments the air in the chamber is mixed continuously by two revolving fans (9 and 10) each of which will move about 30 cb. meters per minute. The blast produced by these fans is rather disagreeable at first but during the work, when the temperature and moisture in the chamber rise, it is felt by the subject as very refreshing. Arrangements for controlling the temperature and moisture have not been put in though they would undoubtedly constitute a considerable improvement. As it is, work experiments can only be made during the cooler season of the year.

The chamber has been tested for tightness by closing it up and connecting it with a spirometer loaded so as to produce a negative pressure of about

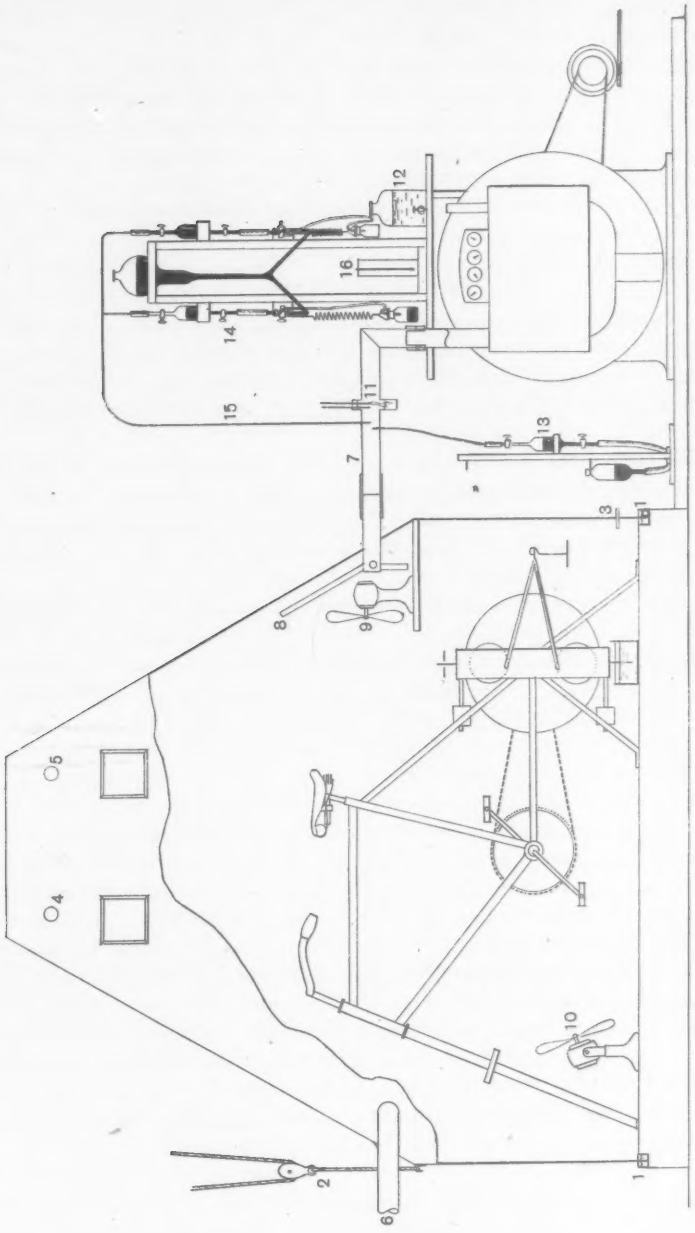

Fig 1.

20 mm. water. No sign of leakage has been detected by such tests. During experiments the negative pressure inside is only about $\frac{1}{2}$ mm. water.

The volume of the chamber has been calculated from numerous measurements of the inside dimensions to 2267 l. This determination was tested by gas analysis in the following way: by means of a 1 litre glass bulb (described by Krogh [1915, 1]) 15·000 l. hydrogen were put into the chamber and thoroughly mixed with the inside air from which 15 l. had been previously withdrawn. From analyses made with the new gas analysis apparatus the volume of the chamber was found to be 2276 l. The volume of the empty chamber is taken therefore to be 2270 l.

In the tubing between the chamber and the meter dry and wet bulb thermometers (11) are arranged and we have satisfied ourselves by repeated comparisons with instruments inside the chamber that these thermometers show accurately within a few tenths of 1° the average temperature and moisture of the air in the chamber—at the rates of ventilation and mixing employed by us.

The gas meter, which produces and measures the air current through the chamber, is a 50 candle wet meter with a drum of 4 cb. feet. It is driven by a small motor ($\frac{1}{10}$ horse power). Through electrical resistances and suitable transmissions the revolutions can be regulated between about 1 in 3 minutes and 3 per minute. When set at a certain speed the motor is controlled by a regulator which will keep the rate constant, generally within $\frac{1}{2}$ % and always within 1 %.

A constant water level is maintained in the meter by a slow current of water passing through it. Water is admitted from the 3 l. bottle (12) and flows off through an overflow tube. The rate at which water is admitted is about $\frac{3}{4}$ l. per hour and care has always been taken not to begin any measurements before the inflow and outflow of water were approximately equal.

As we had to use the meter at the rather rapid rate of nearly 2 revolutions per minute special care has been bestowed upon its calibration at various rates. The results are given in the separate paper by Krogh [1920, 2] and here it is sufficient to mention that at all the rates used the volume per revolution has been determined and can be kept constant within 0·1 %.

Sampling of the air. The atmospheric air entering the chamber has been sampled during each experiment. Sometimes we have taken two separate samples during short periods at the beginning and end of the experiment, but as the differences found between these were always extremely slight we have later taken one sample of 120 cc. in portions of about 20 cc. at more or less regular intervals during the course of the experiment. Details about the composition and constancy of the atmospheric air as taken from a Copenhagen street are given by Krogh [1919].

The outgoing air has been sampled in two ways. At the beginning and end of each experimental period, which is normally of 20 minutes duration, we have drawn a sample by hand in a sampling vessel of about 100 cc.

capacity (13). The taking of such an "intermediate sample" occupies about 1 minute. During the course of each experimental period an "average sample" has been drawn automatically by means of the apparatus shown at (14) which was employed in Greenland by A. and M. Krogh [1913] for a similar purpose. The mercury from the sampling vessel flows off into a bottle suspended by a spring which is so adjusted as to maintain constant within 1 cm. the vertical distance between the surface of the mercury in the sampling vessel and the point of the tube through which it flows out. The volume of air taken into the sampling vessel per minute is therefore practically constant, and as the volume transported by the meter is constant also the sample will represent accurately the average composition of the air even though this should vary considerably. During experiments with constant work the variations are extremely slight. The tubing connecting the sampling vessels with the pipe (7) is of 1 mm. bore. The volume of the tube (15) is about 1·2 cc. and as about 6 cc. are drawn per minute the sample is therefore 12 seconds behind the meter.

Analysis and calculation of experiments. The air samples have been analysed for CO_2 and O_2 in the special gas analysis apparatus constructed for the purpose and described in a separate paper [Krogh, 1920, 1] where the methods of calculating the increase in CO_2 percentage and the O_2 deficit in the outgoing air as well as the correction on the oxygen deficit, which is necessary when the ingoing and outgoing volumes of air are not identical, have also been given in detail and illustrated by an example. The atmospheric air has in all experiments been analysed in duplicate, and in all the experiments made in 1917 the average samples were likewise analysed in duplicate. As the double analyses practically never differed more than 0·001 % we have in the later series usually analysed only a single one of the average samples in duplicate.

Calculation of the gas exchange. We have in all cases taken experimental periods of the length of a whole number of revolutions of the meter (usually 38). By multiplication of the volume per revolution with the number and reduction to 0°, 760 mm. and dryness from the temperature of the meter (shown by the thermometer (16) placed in the outgoing current of air) and the barometric pressure, we obtain the reduced ventilation during the period, which is multiplied by the CO_2 increase and the corrected O_2 deficit found in the corresponding average sample.

As the volume of air which has passed through the chamber during a period is only about double the volume of the chamber itself we cannot take the composition of air in the chamber as unaltered but have to subtract the CO_2 increase and O_2 deficit in the chamber at the beginning of the period and add the corresponding figures for the end of the period. The volume of the chamber (– the ergometer, subject etc.) is therefore calculated for the beginning and end of each period and reduced to 0°, 760 mm. and dryness from the readings of the dry and wet bulb thermometers (11) and the barometric

pressure. The water vapour tension corresponding to the dry and wet bulb readings is taken by interpolation from a psychometric table in Landolt-Börnstein's tables.

From the corrected gas exchange for the experimental period the gas exchange per minute is calculated by division with the length of the period and the respiratory quotient is made out. Finally the total heat production per minute is calculated from the oxygen intake and the respiratory quotient by means of Zuntz's table [1901] disregarding the protein metabolism.

A detailed example of the calculation is given below. The working out of the formulas on which the calculation of respiration experiments with intermediate samples is based is given in detail in the paper by A. and M. Krogh [1913] quoted above.

Rest experiments. In order to calculate the coefficients of efficiency in the muscular work experiments the standard metabolism of the subject must be determined and subtracted from the metabolism during the work periods. It would have been desirable to make these determinations also in a chamber, but for technical reasons this was impossible and we have therefore made them by means of the technique usually employed by us involving the use of a mouthpiece and respiration valves.

The subject who had taken no breakfast sat down in an easy chair on arriving in the laboratory and remained absolutely at rest for 20 minutes. Thereupon the mouthpiece and noseclip were put on, and the subject breathed for about 10 minutes through the valves and expired through a mixing vessel of about 3 l. capacity and a wet meter of 14·50 l. per revolution. During this period the duration of 1 revolution of the meter was observed repeatedly and when it had become approximately constant the determinations were begun. Each experimental period lasted for approximately 2 revolutions of the meter and was begun and finished at the end of an expiration. Two consecutive determinations were made each day. During each a sample of the expired air from the mixing vessel was taken into a 40 cc. sampling vessel of the type usually employed by us [Krogh, 1915, 1]. The sample was drawn by hand, about 2 cc. at a time. One sample of the air inspired from the room was taken in small portions during the two experimental periods. The analyses were reliable to about 0·01 %.

The general routine of making and calculating the experiments is best shown by an example taken from the protocol.

April 19, 1918. The subject O. H. arrived and sat down at 9 o'clock. Mouthpiece etc. at 9.22. Barometer 751·5 mm.

As soon as possible after the determinations of the standard metabolism the subject entered the respiration chamber and began riding the ergometer at 9.45. During the first nine minutes the chamber is not ventilated, to allow the inside air to attain the composition which will remain nearly constant during the whole of the experiment. The subject rides for about half-an-hour before the determinations begin, and during this time the general accommoda-

Rest experiment begun	Duration	Vol. expired l.	Temp. of meter	Samples	Atm. sample
9.31	4′ 27·3″	28·95	17·0°	1	3
	4′ 42·0″	29·20	17·0°	2	

Analyses:

	cc.		%				Expired %	Inspired	Corr.	Diff.
3.	9·988	0·008	0·08 CO_2	1.	9·996	0·395	3·95	0·08		*3·87*
	9·980				9·601	1·670	16·71	20·90 + 0·08		*4·27*
	7·893	2·087	20·90 O_2		7·931		20·66			
			20·98				20·98			
							0·265 × 0·32 = 0·08			
				2.	9·990	0·391	3·91	0·08		*3·82*
					9·599	1·666	16·68	20·90 + 0·10		*4·32*
					7·933		20·59			
							20·98			
							0·265 × 0·39 = 0·10			

Calculation:

	Duration m.	Ventilation per m. L. reduced	CO_2 increase %	O_2 deficit %	R.Q.	Per minute cc. CO_2	Per minute cc. O_2	Calories
1	4·455	5·81	3·87	4·27	0·905	225	248	1·23
2	4·70	5·67	3·82	4·32	0·885	216	245	1·215

tion of the subject to the work, of the temperature in the chamber, of the water level of the meter etc. will take place.

Exp. begun	Duration	Reading of meter	Ventilation revolutions	Temperature of chamber dry	Temperature of chamber moist	Temp. of meter	Samples Intermediate	Samples Average
10.16		9003		18·0°	14·4°	13·2°	1	
	20′ 44·5″		38					A
10.37		9041		19·5°	17·0°	13·2°	2	
	20′ 43·0″		38					B
10.58		9079		20·0°	18·4°	13·3°	3	
	20′ 45·3″		38					C
11.18		9117		20·2°	19·0°	13·4°	4	

Barometer 752 mm. Volume of meter per revolution 113·9 l.
Volume of chamber corrected 2170 l.

The analyses are left out as sufficient examples are given in the preceding paper. The CO_2 increases and O_2 deficits found are given in the calculation.

Calculation:

Sample	Barometer – vapour tension mm.	Temp.	Volume reduced l.	CO_2 increase hundredths of 1 %	O_2 deficit hundredths of 1 %	CO_2 increase litre	O_2 deficit litre
1	742	18·0°	1987	56·15	63·75	11·16	12·67
A	740·5	13·2°	4019	56·95	64·7	22·90	26·00
2	739	19·5°	1968	56·75	64·65	11·17	12·73
B	740·5	13·25°	4019	56·9	64·5	22·87	25·92
3	737·5	20·0°	1961	56·95	64·55	11·17	12·66
C	740·5	13·35°	4019	56·8	64·85	22·83	26·07
4	736·5	20·2°	1956	56·9	65·45	11·14	12·81

Period	Duration m.	CO_2 eliminated l.	O_2 absorbed l.	R.Q.	Per minute CO_2 cc.	Per minute O_2 cc.	Cal.
A	20·74	22·91	26·06	0·880	1105	1256	6·22
B	20·72	22·87	25·85	0·884	1104	1248	6·18
C	20·755	22·80	26·22	0·870	1099	1264	6·24

Sources of Error and Accuracy.

The possible sources of error in the respiration experiments are few in number and not *a priori* likely to be serious. The ventilation recorded by the meter can be taken as correct to about 0·1 %, but the reduced volume of the chamber may possibly be erroneous to the extent of 0·2 %, if the recorded temperature and moisture differ from the true averages by $\frac{1}{2}$° and $\frac{1}{2}$ mm. vapour pressure. Such an error will influence the final result to the extent of 0·1 %.

Errors in the analyses may amount to 0·001 % and since the CO_2 percentage is generally about 0·5 % this will correspond to an error on the gas exchange of 0·2 %. If the CO_2 increase should be found, say, 0·001 % too low and the O_2 deficit 0·001 % too high the resulting error on the respiratory quotient would be 0·004.

A priori the possibility cannot be excluded that the samples analysed do not represent accurately the average composition of the air. This may be the case especially with the intermediate samples if the air in the chamber is not absolutely mixed.

To test this point we have made a small number of control experiments. In these we have added CO_2 from a cylinder to the chamber through a 1 litre meter. The CO_2 was washed with permanganate and the water in the meter

Control experiment 1.

Time	Duration seconds	CO_2 %
10.45	52	·5085
	57	·5085
	70	·508
	59	·5015
	70	·511
	9	·509
	31	·5085
	67	·5095
	7	·508
10.55	65	·509
	Average	0·5082
	Dispersion $\sigma = \pm 0{\cdot}0025$	

Control experiment 2.

Time	Duration seconds	CO_2 %
10.38	33	·509
	60	·5105
	10	·510
	90	·513
	7	·5105
	70	·510
	15	·5075
	92	·5095
	16	·5035
	70	·5095
10.51	60	·510
	61	·514
	60	·5115
	13	·510
	55	·5125
11.00	55	·5035
	Average	0·5097
	Dispersion $\sigma = \pm 0{\cdot}0028$	

was thoroughly saturated with the gas before any experiments were made. During the experiments CO_2 was added at an approximately constant rate of 1 l. per minute while the chamber was being ventilated at the rate used in actual work experiments. It is obvious that the CO_2 from a constant slow current of the pure gas is not so likely to become uniformly mixed with the air of the chamber as the expired air from a human subject, which contains only about 5 % CO_2.

The first control experiments consisted simply in taking intermediate samples as rapidly as possible and comparing their CO_2 percentage, which should remain constant.

The dispersions of the single samples do not differ significantly in both series, viz. about 0·5 % of the quantity of CO_2. If the CO_2 percentage in one of the intermediate samples is, say, 1 % too high the CO_2 production in the preceding 20 minutes period will be found 0·5 % too high, since the volume of the chamber is about half the ventilation during 20 minutes, while in the following period it will be 0·5 % too low.

We have made two complete control experiments consisting of five 15 minute periods during which CO_2 was added at an approximately constant rate. The results were as follows:

Control experiment 3.

No.	Duration m.	CO_2 found l.	CO_2 added l.	CO_2 per minute found cc.	CO_2 per minute added cc.	Difference %
1	15·395	14·21	14·33	922	930	−0·9
2	15·42	14·20	14·29	920	926	−0·7
3	15·37	14·28	14·32	928	931	−0·3
4	15·39	14·32	14·31	930	929	+0·1
5	15·355	14·54	14·26	946	928	+1·9
		71·55	71·51			

Control experiment 4.

1	16·54	15·29	15·43	924	932	−0·9
2	15·44	14·41	14·37	933	930	+0·3
3	15·41	14·24	14·37	924	932	−0·9
4	15·44	14·27	14·34	924	928	−0·7
5	15·44	14·26	14·39	924	931	−0·8
		72·17	72·90			

According to these control experiments we have every reason to expect that the error in the respiration experiments on a working subject in which the conditions for complete mixing of the air are distinctly more favourable than in the controls will practically never exceed 1 %.

In order finally to test the accuracy with which the respiratory quotient can be determined we have made a control experiment on a human subject, doing the normal amount of work on the ergometer, while the chamber was ventilated as usual, by taking a continuous series of "intermediate samples." We cannot of course expect that the gas exchange of the subject will remain

constant during the whole period covered by the samples, but as the air in the chamber is only very gradually renewed the possible changes in the composition of the air and the respiratory quotient must obviously be very gradual. Since the air expired from the subject must show the same respiratory quotient whether it be mixed with a small or a large quantity of pure air, the oscillations observed in the respiratory quotient must be due chiefly to errors in the gas analysis, while oscillations in the CO_2 increase coincident with similar oscillations in the O_2 deficit must be due to incomplete mixing. The results were as follows:

Control experiment 5.

Time	Duration seconds	CO_2 increase °/₀₀	O_2 deficit °/₀₀	R.Q.
10.16	61	65·75	70·8	0·929
	69	65·1	70·4	0·925
	68	66·0	71·1	0·928
	91	65·95	71·3	0·925
	62	65·65	70·85	0·928
	47	66·0	70·85	0·932
	75	66·2	71·3	0·929
	83	66·4	71·65	0·927
10.28	70	66·7	71·8	0·930
				Average 0·928
				Dispersion $\sigma = \pm 0{\cdot}002$

The variations in the respiratory quotient correspond closely to the unavoidable errors in the gas analysis, but the percentages of CO_2 and O_2 show somewhat larger variations especially in the second and fifth sample indicating deficiencies in the mixing amounting sometimes to $\frac{1}{2}$ or even 1 % of the CO_2 increase or O_2 deficit.

In the calculation of the energy metabolism from the results of the respiration experiments we have like Benedict and Cathcart [1913] used the method introduced by Zuntz and Schumburg [1901]. We have not used the original table but the latest given by Zuntz and Loewy [1913]. This table which shows the caloric value of oxygen at quotients between 0·71 and 1·00 is partly reproduced on p. 310. It differs slightly from the earlier tables. The use of this table presupposes that anabolic processes do not take place, and it refers only to the catabolism of fats and carbohydrates, assuming that the oxygen and carbon dioxide corresponding to the protein metabolism are subtracted beforehand. Like Benedict and Cathcart, Loewy [1911] and others we have made our calculations on the basis that fats and carbohydrates are exclusively catabolised. It will be necessary to estimate the error resulting from this simplification. Almost all the experiments have been made on low protein diets, but it has not been practicable to collect the urine and determine the protein metabolism.

We can assume a protein metabolism of 75 g. per day as the maximum during our experiments. This corresponds to 12 g. nitrogen or 0·5 g. per hour.

0·5 g. nitrogen per hour requires per minute 49·4 cc. oxygen and produces 0·222 Calorie, while the same quantity of oxygen would when used to burn a mixture of fat and carbohydrate produce 0·242 Calorie. The maximum error committed by disregarding the protein metabolism is therefore 0·02 Calorie or 0·3–0·4 % of the metabolism during work.

The accuracy of the technique adopted for the respiration experiments during rest has been studied and discussed by Liljestrand [1916]. The error on a 5 minute experiment may amount to about 5 % or 10 cc. CO_2 or O_2 per minute. We have made a technical error in taking the samples of expired air from the mixing vessel synchronously with the periods of ventilation. As the volume of the lungs + tubing + mixing vessel is about 6 l. a change in ventilation will not correspond to a simultaneous change in the composition of the air from which the sample is drawn, but this change will on an average take place 6 respired liters or 1 minute later. The taking of samples should therefore have been begun 1 minute after the beginning of each period and continued for 1 minute after the end of it. With an absolutely uniform respiration no error can arise from this source, but the respiration during rest is seldom absolutely uniform. The large number of double determinations made by us furnish material for a statistical study of the accuracy which w be referred to below (Appendix I, p. 346).

The muscular work was in all our experiments performed on the bicycle ergometer constructed by Krogh [1913]. The instrument was provided with automatic control of the current [Krogh, 1915, 3]. A load of 10 g. is sufficient to bring about a contact actuating the adjusting motor, and the maximum error on the determination of the load is therefore 10 g. or 1 % of the load usually employed. When work is being performed it can be observed that the adjusting motor is generally actuated for a moment about every minute, now increasing now diminishing the load. This is due to the unavoidable small irregularities in the pedalling, but these oscillations must in our opinion counterbalance each other so that the final error on the effective load will be much below 1 %.

The rate of pedalling was controlled by a metronome which could be observed by the subject through one of the windows in the chamber. We soon found that it was difficult to control the rate by sight and installed an electric bell actuated by the metronome inside the chamber. After a minute or two a certain phase in the movements of the legs is synchronous with the signal, and the rate is maintained with a minimum of mental effort.

We found that the metronome employed by us would not work with absolute constancy and in most experiments the subject has therefore employed 10 minutes during each of the experimental periods in controlling the metronome by counting and comparing with a watch. This has the further advantage of relieving somewhat the monotony of the work. The maximum variation observed in the rate of the metronome is 0·4 %.

In preliminary tests with each subject the load and rate of pedalling were

adjusted to the muscular strength and the convenience of the subject. Some preferred a rate of 60 revolutions per minute, but most of the experiments were done at the rate of about 50.

The subject always began to work just after entering the chamber and worked for half-an-hour before the experiments began. We have repeatedly made determinations of 10–20 minutes duration during this introductory period, but we have found that the results were distinctly less regular than they became later and these determinations have been left out of account in the working out of the results.

We have made several separate series of experiments and after each series we have reviewed the results secured and the technique employed to find out whether by suitable changes in the arrangement we might hope to avoid some of the irregularities encountered. As this is, in our opinion, one of the most important points in the research we propose to report the experiments in the order in which they were made, to set forth the reasons for the changes in routine adopted and discuss their results.

Preliminary Series of Experiments.

Our first plan was worked out in collaboration with Dr G. Liljestrand of Stockholm who took part also in this series of experiments. We would make determinations on four subjects and would act alternately as subjects ourselves. On each subject we would make a preliminary experiment which should not be included in the series. After a suitable interval the subject should take a diet consisting either chiefly of carbohydrates or chiefly of fats. In the first experiments (on J. L. and G. L.) no precautions were taken regarding protein, but later it was provided that the experimental diets should contain a minimum of protein. The diet should be taken for two consecutive days and on the morning of the third day an experiment should be made before the subject took any food. During the third day the diet should be continued and a second experiment should be made on the morning of the fourth day. Each experiment should consist of 2 hours' work with 3 half-hour periods in which the metabolism should be determined. Thereupon the subject should have a rest of several days before taking the alternative diet. We hoped by taking two days' diet before the experiments to obtain very high or respectively very low quotients. It was arranged further that in the fat periods the subjects should take a certain amount of exercise to get rid of as much glycogen as possible.

Two of the subjects should begin with the carbohydrate diet and two with the fat diet, and we hoped in this way to neutralise the possible influence of training.

The technique was during these preliminary experiments not so elaborate and precise as it became later. The automatic device on the ergometer was not regularly controlled and the metronome was assumed to work at a constant rate when once set and was controlled only at long intervals. As the rate was

found to have changed distinctly we had to obtain the rates in the experiments done during the interval by interpolation. The motor driving the gas meter was not provided with a regulator and its rate varied about 3 %. The revolutions of the meter were counted by an electrical device which proved not to be absolutely reliable. The length of the experimental periods was regulated by the rate of outflow of the mercury from the sampling vessels which differed somewhat in size; they varied therefore between 22 and 32 minutes. In a few cases, which will be duly noted, these imperfections may have caused errors in the determinations but usually their influence is imperceptible.

Determinations of the metabolism during rest were not made regularly on the first two subjects (J. L. and G. L.) but the standard metabolism must be deduced from 2–3 experiments.

Experiments were made on the following subjects:

J. L. Age 46 years. Weight 67 kg. Height 171 cm. Practised in bicycling but not specially trained.

G. L. Age 31 years. Weight 51 kg. Height 166 cm. Had very little practice in bicycling.

A. K. Age 42 years. Weight 67 kg. Height 176 cm. Practised in bicycling but not trained.

R. E. Age 25 years. Weight 64 kg. Height 170 cm. In moderate training as a bicyclist.

From the notes made by the subjects the following points are selected:

J. L. Fat diet, Jan. 13th to 15th (inclusive). Work on the ergometer on the 15th and 16th. The diet consisted chiefly of pork, eggs and meat with cabbage and butter. Some claret was taken at meals. The subject took plenty of food and had no digestive trouble.

Exercise: a 2–3 hours' walk on the 13th and 14th. Very tired afterwards. Indisposition with fever and headache began on the 14th and continued throughout the period. Body temperatures: 15th morning, 37·5°, night, 38·2°; 16th, 36·7°, 37·4°; 17th, night, 39·2°; 18th, 38·0°, 37·0°; 19th, 36·0°, 37·0°. Pulse rate: 15th, 77; 17th, 90 and 19th, 58 which latter figure is normal for the subject.

Work on the ergometer on the 15th. Moderate perspiration, tired afterwards. On the 16th, profuse perspiration, work performed with great difficulty. Extremely tired during the afternoon.

Carbohydrate diet, Jan. 30th to Feb. 1st. The diet consisted chiefly of porridge, bread, cakes, sugar, honey, marmalade, green vegetables.

No particular exercise was taken.

Work on the ergometer on Feb. 1st and 2nd, performed with ease.

G. L. Carbohydrate diet, Jan. 16th to 18th, same as for J. L. A meal of cakes was taken late in the evening on the 17th and 18th. No exercise.

Work on the ergometer 18th and 19th performed without difficulty.

Fat diet, Jan. 23rd to 25th, same as for J. L.

Exercise: about two hours' walk on the 23rd and 24th.

Table II.

Series I. General Table of Experiments.

	Rest				Work					
Date and subject	Vent. l. per min. 0°, 760 mm.	O_2 per min. cc.	R.Q.	Cal. per min.	Length of per.[1]	O_2 per min. cc.	R.Q.	Cal. per min.	Revolut. per min.	Techn. work Cal. per min.
J. L.				*c*				*b*	*a*	
15. i.					32	1220	0·772	5·92		
					30	1256	0·756	6·07	60·2[2]	0·918
					29	1316	0·760	6·46		
16. i.					27	1256	0·767	6·08		
					24	1247[3]	0·771	6·05	60·3[2]	0·920
					22	1265	0·791	6·16		
1. ii.	4·03	218	0·77	1·055	26	1156	0·846	5·68		
	4·11	216	0·81	1·055	25	1151	0·873	5·68	61·3[2]	0·935
					26	1163	0·868	5·74		
2. ii.	3·99	203	0·81	0·995	28	1187	0·846	5·84		
	3·92	205	0·80	1·00	26	1194	0·843	5·87	61·5[2]	0·937
					28	1186	0·836	5·82		
G. L.										
18. i.					25	860	0·906	4·28		
					29	898	0·885	4·45	60·5[2]	0·645
					28	902	0·867	4·45		
19. i.					32	944	0·927	4·72		
					28	936	0·913	4·66	60·7[2]	0·647
					29	903	0·900	4·48		
25. i.	5·17	228	0·765	1·10	31	957	0·766	4·63		
	5·29	229	0·765	1·11	24	970	0·777	4·71	61·0[2]	0·650
					26	1014	0·751	4·90		
26. i.	4·80	216	0·765	1·045	28	989[4]	0·762	4·79		
	4·89	210	0·80	1·025	29	995	0·757	4·81	61·1[2]	0·651
					28	1009	0·762	4·88		
26. ii.	5·32	212	0·80	1·035	28	931	0·863	4·59		
	4·43	196	0·79	0·955	30	971	0·857	4·78	62·3	0·663
					23	954	0·833	4·68		
A. K.										
12. ii.	6·50	209	0·875	1·035	27	1053	0·896	5·22		
	6·60	213	0·87	1·05	26	1070	0·867	5·28	61·7[2]	0·940
					28	1087[5]	0·861[5]	5·36[5]		
13. ii.	6·51	215	0·88	1·065	27	1081	0·909	5·38		
	6·68	206	0·94	1·03	27	1090	0·893	5·41	61·8	0·941
					29[6]	1132	0·883	5·60		

[1] The length of the period has been given in whole minutes.

[2] Revolutions obtained by interpolation from countings of metronome on the 13th Jan. and 13th Feb. and following days.

[3] Recorded revolutions of meter 46, corrected according to the rate in the first and third period to 47. (See also notes 4 and 6.)

[4] Counter on meter failed. Number of revolutions calculated from ventilation in second and third period.

[5] The last "intermediate" sample of air was lost. As the composition of the air in the preceding samples was approximately constant the composition was assumed in accordance with these.

[6] Counter on meter was seen to fail just before the reading.

Table II *continued.*

	Rest				Work					
Date and subject	Vent. l. per min. 0°, 760 mm.	O_2 per min. cc.	R.Q.	Cal. per min.	Length of per.[1]	O_2 per min. cc.	R.Q.	Cal. per min.	Revolut. per min.	Techn. work Cal. per min.
A. K.				*c*				*b*	*a*	
19. ii.	6·96	254	0·725	1·22	28	1094[2]	0·781	5·31[2]		
	6·14	215	0·75	1·04	28	1134	0·774	5·50	61·9	0·941
					28	1135	0·755	5·49		
20. ii.	6·88	241	0·70	1·15	26	1221	0·757	5·90		
	6·91	246	0·73	1·185	26	1238	0·762	5·99	62·0	0·945
					26	1255	0·757	6·07		
1. iii.	6·18	225	0·83	1·10	22	1068	0·891	5·30		
	6·20	222	0·885	1·10	30	1122	0·870	5·54	62·4	0·950
					22	1145	0·846	5·63		
R. E.										
20. iii.	5·59	234	0·85	1·15	31	1092	0·854	5·38		
	5·44	233	0·83	1·14	30	1096	0·836	5·38	49·8	0·986
					32	1112	0·817	5·44		
25. iii.					29	1210	0·747	5·84	49·8	0·986
	6·09	290	0·73	1·395	30	1308	0·742	6·30	?	?
	5·61	276	0·71	1·32	29	1406	0·751	6·79	(57·2)[3]	(1·13)
26. iii.	5·48	258	0·745	1·245	28	1194	0·742	5·76		
	5·48	264	0·76	1·275	27	1221	0·730	5·87	49·8	0·986
					29	1250	0·738	6·02		
6. iv.	6·40	269	0·93	1·34	29	1054	0·891	5·23		
	6·05	263	0·90	1·305	26	1052	0·895	5·22	49·9	0·988
					30	1056	0·879	5·23		
7. iv.	5·71	250	0·93	1·25	30	1064	0·932	5·32		
	5·91	253	0·95	1·27	25	1086	0·918	5·41	49·9	0·988
					31	1140[4]	0·897	5·66[4]		

[1] See note 1 on preceding page.

[2] See note 4 on preceding page.

[3] Several countings showed that the subject was not keeping time with the metronome.

[4] Duration of last period slightly uncertain. The figure for the metabolism possibly 1 % too high.

Felt very tired on the 24th.

Work on the ergometer 25th and 26th performed with considerable difficulty. After the work the subject was very tired.

A. K. Carbohydrate diet, Feb. 10th to 12th. Food: potatoes, flour, bread, cakes, marmalade, sugar. A meal of cakes and marmalade with a little alcohol taken late (about 11 p.m.) on the 11th and 12th. No special exercise.

Work on the ergometer 12th and 13th performed with ease. No perspiration.

Fat diet, Feb. 17th to 19th. Very fat bacon, cream, butter, eggs, and cabbage. Plenty of food taken on the 17th and 18th. Less on the 19th. Some diarrhoea on the 18th.

Exercise: 3½ hours' walk (about 18 km.) on the 18th. Felt tired afterwards.

Work on the ergometer 19th performed with some difficulty. Rather

tired afterwards. On the 20th work carried through with extreme difficulty. Very tired for several hours afterwards.

R. E. Fat diet, March 22nd to 25th. Food: fat bacon, butter, cream, cabbage. Three meals a day. Felt hungry between meals

Exercise: 1 hour's walk and a little gymnastics each day.

Work on the ergometer 25th and 26th performed without difficulty.

Carbohydrate diet, April 4th to 6th. Food: bread, cakes, apples, potatoes and green peas. No special exercise.

Had very little sleep between the 5th and the 6th.

Work on the ergometer on the 6th. Felt tired and sleepy. Work appeared difficult. 7th, work easier but apparently not easier than on the fat diet.

An inspection of the columns R.Q. and Cal. in the general table shows an unmistakeable tendency for the amount of energy, expended to perform a practically constant amount of muscular work, to vary inversely as the quotient, which means that the energy value of fat and carbohydrate for work is not the same. The value of fat is smaller than that of carbohydrate.

The difference in value for muscular work of fat and carbohydrate can be expressed quantitatively either as a difference in percentage muscular efficiency or as a difference in the number of Calories expended to perform 1 Calorie of external work. Representing by a the output of external work in Calories per minute, by b the total amount of energy expended per minute, while doing the work, and by c the energy output per minute during rest we have the percentage "gross efficiency" $\frac{100a}{b}$ and the corresponding "net efficiency" $\frac{100a}{b-c}$, while the corresponding expenditure of Calories per unit work are gross $\frac{b}{a}$ and net $\frac{b-c}{a}$. There is a general agreement that valid comparisons can only be obtained by using "net" values, but certain differences of opinion exist about the most correct definitions for a and c.

Lindhard [1915] has shown in detail that the amount of work recorded by a bicycle ergometer is considerably lower than the total amount actually performed in riding. The real efficiency is therefore greater than the apparent or "technical" efficiency obtained from the amount of work directly recorded. Since, however, we have to deal with differences only it does not much matter for our purposes whether the efficiencies calculated by us are true or only technical efficiencies, provided we are justified in assuming that there is a constant or approximately constant relation between the technical and the true efficiency. It has been shown by Lindhard [1915] that the relation between the true and the technical efficiency varies with the rate of pedalling and also to a certain extent with the load, but we have every reason to believe that when both the load and the rate are kept constant throughout each series of determinations the use of technical efficiencies for comparison purposes will give correct results.

A further point of difficulty and controversy in calculating efficiencies or expenditure of Calories per unit work is the choice of a proper value for c,

the "base line" of the work. Benedict and Cathcart [1913] have made calculations of efficiencies from several different base lines, viz. complete rest, rest in a sitting posture on the ergometer, riding on an ergometer without load and riding on an ergometer which was revolved by a motor while the legs of the subject followed the motion passively. Lindhard [1915] has criticised these base lines and shown that complete rest and rest in the position in which the work is performed furnish the most reliable base lines. In our case we must expect that any difference between the value of carbohydrates and fats will exercise its influence equally upon all kinds of muscular work and also on the work of maintaining a certain posture on the ergometer, and it follows therefore that we must take complete rest as our logical base line.

The question remains as to whether the rest experiments of each day should be used for combination with the work experiment of the same day or whether a large number of rest experiments should be averaged and the average standard metabolism used as base line for all the work experiments. Lindhard [1915] has pointed out that if the variation in the rest experiments is purely accidental there is no reason to suppose that the same accidental factors (*e.g.* slight movements of the subject, small technical errors in the determination) will influence the work experiments of the same day in the same direction, and it will therefore be safer and give more correct results to average all the available determinations made during rest. In our experiments it is possible, though *a priori* not very probable, that the variations in diet may have an influence upon the resting metabolism. If that should be the case rest experiments on a certain diet should be used as base line for work experiments on the same diet.

In the above tables the rest experiments are too few in number to decide whether the diet has any influence or not upon the standard metabolism, though there is in the experiments on A. K. and R. E. some indications of a slightly increased metabolism when the quotient is very low. We have therefore averaged the rest experiments done on each subject and used the averages as base lines c in the calculations mentioned above.

When we began working out the results of this research we calculated "net efficiencies" $\frac{100a}{b-c}$ in accordance with the principles indicated above, but we found later that the reciprocal values $\frac{b-c}{a}$ (expenditure of energy per unit work) were better suited for the comparisons between the value of fat and carbohydrate. What we want to find is the difference in value between fat and carbohydrate or the waste of energy resulting from the combustion of fats. Putting $b - c = d$ we have, when fats alone are the source of energy during muscular work, the expenditure of energy per unit work $e_F = \frac{d_F}{a}$ and for carbohydrates $e_C = \frac{d_C}{a}$. The difference between these figures $e_F - e_C = w$ represents the waste of energy from fat and $\frac{100w}{e_F}$ the percentage waste of energy.

As we are unable to obtain experimentally a catabolism exclusively of fat (R.Q. = 0·71) or exclusively of carbohydrate (R.Q. = 1·00) and may obtain all possible quotients between these limits it becomes necessary to make out the quantitative relation between the respiratory quotient and the relative amounts of fat and carbohydrate catabolised. Table III, which is calculated from the corresponding table of Zuntz, shows for quotients varying from 0·71 to 1·00 the relative amounts of energy derived from carbohydrate and fat respectively.

Table III.

R.Q.	Energy value of 1 l. O_2 Cal.	Cal. from carbohydrate	Cal. from fat	Per cent. energy from fat	$\frac{100\,(1 - \text{R.Q.})}{1{\cdot}00 - 0{\cdot}71}$
0·71	4·795	0	4·795	100	100
0·75	4·829	0·647	4·182	86·6	86·3
0·80	4·875	1·530	3·345	68·6	69·0
0·85	4·921	2·411	2·510	51·0	51·8
0·90	4·967	3·295	1·672	33·65	34·5
0·95	5·012	4·175	0·837	16·7	17·2
1·00	5·058	5·058	0	0	0

The calculation shows that the percentage amount of energy derived from fat is approximately a straight line function of the respiratory quotient and can be expressed by the formula $\frac{100\,(1 - \text{R.Q.})}{1{\cdot}00 - 0{\cdot}71}$.

If, therefore, we take as our theoretical basis the hypothesis that the energy output per unit work can be expressed as the sum of a certain amount of energy derived from carbohydrate, which can be utilised "directly," and another amount derived from fat, which can only be utilised after a "conversion" involving the loss of a definite fraction of the energy, we can deduce as consequences of this hypothesis (1) that the curve expressing the relation between the energy output per unit work $\frac{d}{a} = e$ as ordinate and the respiratory quotient as abscissa should be a straight line and (2) that the percentage waste of energy from fat should be the same for different subjects.

The percentage waste of energy from fat $\frac{100\,(e_F - e_C)}{e_F}$ can be found from any two points of the curve by computation and graphically by extrapolation of the curve to the quotients 0·71 and 1·00. When we have the energy per unit work at the quotient q_1 to be e_1 and at the quotient q_2 to be e_2 we get

$$e_F - e_C = \frac{e_1 - e_2}{q_2 - q_1}\,0{\cdot}29 \text{ and } e_F = e_1 + \frac{e_1 - e_2}{q_2 - q_1}\,(q_1 - 0{\cdot}71).$$

In Table IV the determinations on each subject are arranged in series according to the quotients. By means of the dates and numbers given each period can be referred back to the general table. In the figures 2–5 the results have been plotted with the quotients as abscissae and the expenditure of energy per unit work e as ordinates. The first periods of each experiment have been denoted by ×, the second by • and the third by o. It is seen that the third periods deviate in certain cases and notably at low quotients from

Table IV.

Date and number	R.Q. work	Work Cal.	Rest Cal.	Difference	Cal. per unit of work	Technical work Cal.	Deviation from curve
J. L.		*b*	*c*	*d*	*e*	*m*	*δ*
15. i. 2	·756	6·07	1·03	5·04	5·49	0·918	
15. i. 3	·760	6·46		5·43	5·91	0·918	
16. i. 1	·767	6·08		5·05	5·49	0·920	
16. i. 2	·771	6·05[1]		5·02	5·46	0·920	
15. i 1	·772	5·92		4·89	5·33	0·918	
16. i. 3	·791	6·16		5·13	5·58	0·920	
2. ii. 3	·836	5·82		4·79	5·12	0·937	
2. ii. 2	·843	5·87		4·84	5·17	0·937	
1. ii. 1	·846	5·68		4·65	4·97	0·935	
2. ii. 1	·846	5·84		4·81	5·14	0·937	
1. ii. 3	·868	5·74		4·71	5·04	0·935	
1. ii. 2	·873	5·68		4·65	4·97	0·935	
G. L.							
25. i. 3	·751	4·90	1·04	3·86	5·94	0·650	+0·2
26. i. 2	·757	4·81		3·77	5·79	0·651	+0·07
26. i. 1	·762	4·79		3·75	5·76	0·651	+0·05
26. i. 3	·762	4·88		3·84	5·90	0·651	+0·19
25. i. 1	·766	4·63		3·59	5·52	0·650	−0·18
25. i. 2	·777	4·71		3·67	5·64	0·650	−0·03
26. ii. 3	·833	4·68		3·64	5·49	0·663	−0·03
26. ii. 2	·857	4·78		3·74	5·64	0·663	+0·18
26. ii. 1	·863	4·59		3·55	5·36	0·663	−0·08
18. i. 3	·867	4·45		3·41	5·29	0·645	−0·15
18. i. 2	·885	4·45		3·41	5·29	0·645	−0·10
19. i. 3	·900	4·48		3·44	5·32	0·647	−0·03
18. i. 1	·906	4·28		3·24	5·02	0·645	−0·32
19. i. 2	·913	4·66		3·62	5·60	0·647	+0·28
19. i. 1	·927	4·72		3·68	5·69	0·647	+0·41
						Sum	+0·47
A. K.							
19. ii. 3	·755	5·49	1·10	4·39	4·67	0·941	−0·25
20. ii. 1	·757	5·90		4·80	5·08	0·945	+0·16
20. ii. 3	·757	6·07		4·97	5·26	0·945	+0·34
20. ii. 2	·762	5·99		4·89	5·18	0·945	+0·28
19. ii. 2	·774	5·50		4·40	4·68	0·941	−0·19
19. ii. 1	·781	5·31[1]		4·21	4·48	0·941	−0·37
1. iii. 3	·846	5·63		4·53	4·77	0·950	+0·09
12. ii. 3	·861	5·36		4·26	4·53	0·940	−0·11
12. ii. 2	·867	5·28		4·18	4·45	0·940	−0·18
1. iii. 2	·870	5·54		4·44	4·68	0·950	+0·06
13. ii. 3	·883	5·60		4·50	4·78	0·941	+0·19
1. iii. 1	·891	5·30		4·20	4·42	0·950	−0·14
13. ii. 2	·893	5·41		4·31	4·58	0·941	+0·02
12. ii. 1	·896	5·22		4·12	4·38	0·940	−0·17
13. ii. 1	·909	5·38		4·28	4·55	0·941	+0·03
						Sum	−0·24
R. E.							
26. iii. 2	·730	5·87	1·27	4·60	4·67	0·986	+0·02
26. iii. 3	·738	6·02		4·75	4·82	0·986	+0·20
26. iii. 1	·742	5·76		4·49	4·55	0 986	−0·06
25. iii. 1	·747	5·84		4·57	4·63	0·986	+0·04
20. iii. 3	·817	5·44		4·17	4·23	0·986	−0·12
20. iii. 2	·836	5·38		4·11	4·17	0·986	−0·11
20. iii. 1	·854	5·38		4·11	4·17	0·986	−0·05
6. iv. 3	·879	5·23		3·96	4·01	0·988	−0·13
6. iv. 1	·891	5·23		3·96	4·01	0·988	−0·09
6. iv. 2	·895	5·22		3·95	4·00	0·988	−0·08
7. iv. 3	·897	5·66		4·39	4·44	0·988	+0·36
7. iv. 2	·918	5·41		4·14	4·19	0·988	+0·19
7. iv. 1	·932	5·32		4·05	4·10	0·988	+0·15
						Sum	+0·32

[1] Slightly uncertain.

the rest and give higher values for *e*. This must be ascribed to the fatigue which was repeatedly very pronounced.

It is obvious that the experiments are too few and too discordant to allow of a quantitative estimate of the energy waste from fat. It is possible to draw

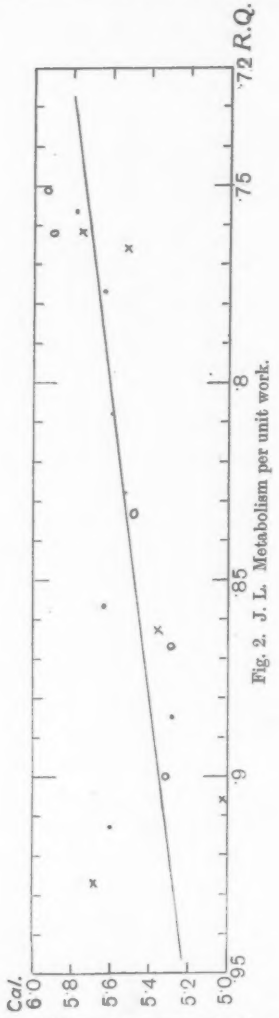

Fig. 2. J. L. Metabolism per unit work.

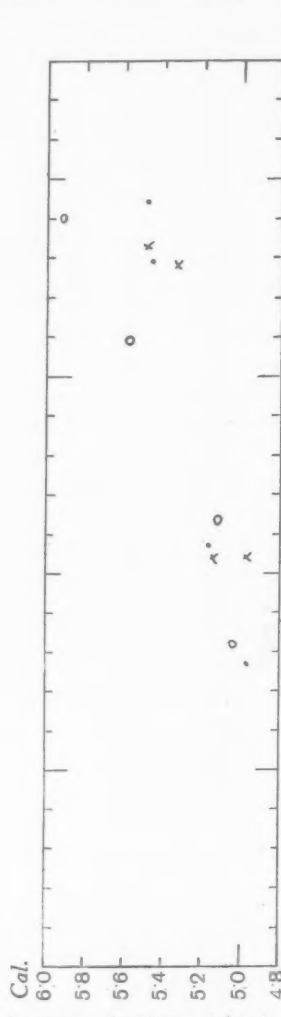

Fig. 3. G. L. Metabolism per unit work.
× First experimental period, • second, o third period.

rather different curves which will suit the determinations at least as well as the straight lines which we have drawn. In the case of J. L. we have not drawn any curve. On account of his illness during the fat period we think it better to disregard the experiments made on this subject.

We have measured the vertical distance (in Cal. per unit work) of each point in Figs. 2–5 from the curve to which it belongs and these distances have been given in the last column of Table IV as the deviation from the curve (δ). If the curves were correctly drawn and all the determinations were

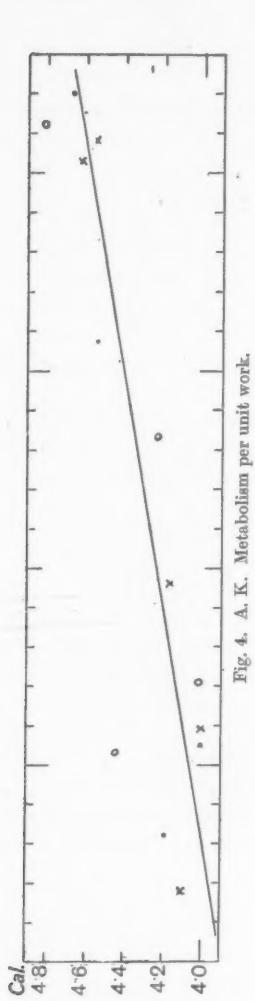

Fig. 4. A. K. Metabolism per unit work.

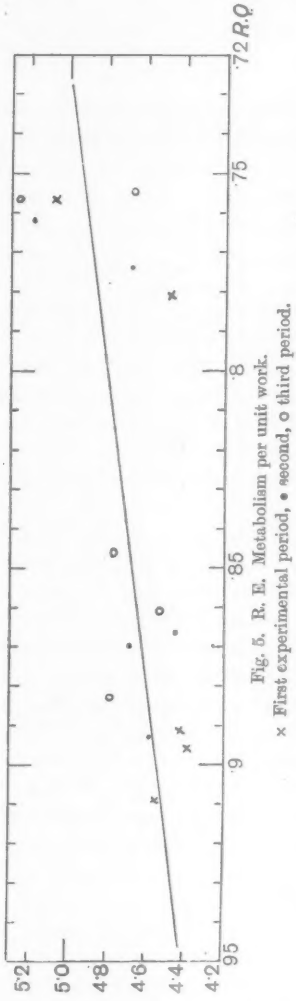

Fig. 5. R. E. Metabolism per unit work.
× First experimental period, • second, o third period.

of equal weight the algebraic sum of these deviations should be 0. From the deviations given the dispersion (σ) or "standard deviation" has been calculated for each curve according to the formula $\sigma = \sqrt{\frac{\Sigma\delta^2}{n-1}}$ and we have further

calculated the dispersion in per cent. of the average metabolism per unit work $e_{0\cdot85}$. We find

Table V.

	G. L.	A. K.	R. E.
n	15	15	13
$e_{0\cdot85}$ Cal.	5·48	4·17	4·24
σ Cal.	0·20	0·21	0·155
σ %	3·6	4·4	3·7

When the waste of energy is calculated from the straight lines drawn we obtain the following results:

Table VI.

	G. L.	A. K.	R. E.
e_F Cal.	5·84	5·04	4·72
e_C Cal.	5·09	4·28	3·72
Waste from fat Cal.	0·75	0·76	1·00
Waste %	12·9	15·1	21·2
Efficiency on carbohydrate $\frac{100}{e_C}$	19·6	23·4	26·8

The low efficiency of the subject G. L. is probably to be explained by his want of training as a bicycle rider.

The results are obviously of very limited value quantitatively and it should be specially pointed out that the exercise taken during the fat periods and not during the carbohydrate periods may constitute a systematic error.

Second Series of Experiments.

As a result of the preliminary series of experiments several improvements were introduced into the technique and routine. The purely technical improvements have been alluded to above. As we ourselves were evidently not suitable as subjects, partly because our other duties made it impossible for us to lead an absolutely uniform life during the experimental period, we resolved to secure the assistance of an intelligent student who should be, as far as possible, a trained athlete both generally and as a bicyclist, hoping thereby to minimise the influence of training. We would further shorten the dietary periods and make a larger number of determinations also at intermediate quotients to obtain data for determining the form of the efficiency curve. The length of the experimental periods should be somewhat reduced to minimise the influence of fatigue.

We were very fortunate in securing the services as subject of Mr Olaf Hansen a student of languages and gymnastics who possessed all the qualifications desired.

His age was 23 years, weight 80 kg. and height 179 cm. He is a trained athlete and was in good form at the time but lately he had practised bicycling to a very limited extent only. He was carefully instructed with regard to the experimental routine, and the most suitable load on the machine and rate of pedalling were found by trials. No hint whatever was given of the

subjective results expected from the different diets, but the subject was asked to make notes both of the food eaten and of the influence of the work upon his well-being and physical fitness. From these notes, which gave very full information, we have made the following extract.

From May 6th (1917) to 11th mixed diet, poor in protein. Went for a walk of 7 km. on the 6th. Otherwise no exercise. Work on the ergometer 7th, 8th, 10th and 11th. Not the least tired after each day's work. Weight on the 9th, 80 kg.

Carbohydrate diet 12th to 18th. Three meals a day on the experiment days, otherwise four. Last meal in the evening between 7 and 10 o'clock. Food chiefly porridge, bread, potatoes and other vegetables, sugar, honey, marmalade and fruit. Exercise: walk of 7 km. on the 13th, cycling about 17 km. on the 17th. This tour was taken in the afternoon after work on the ergometer in the morning. No fatigue whatever was felt. Work on the ergometer 14th, 15th, 16th and 17th performed with very slight perspiration and not in the least fatiguing. The work was felt to be easier than in the preceding week and on the 14th the subject asked if the load had not been diminished. Weight on the 16th, 79·5 kg.

Fat diet 19th to 24th. On the experiment days a cup of tea without sugar or milk was taken in the morning and large meals at 12 noon and 7 night. Cocoa and cream in the afternoon at 3. Food: ham, eggs, bacon, butter, cream and green vegetables. Exercise: cycling about 38 km. on the 20th in slow time, 25 km. on the 23rd in the evening; felt tired in the legs especially during first half of the tour. No experiments on these days. Cycling about 9 km. on the 24th, in the evening after an experiment. Felt very tired during the whole week and could not do the usual amount of mental work. Work on the ergometer 21st and 22nd. Perspiration profuse, extremely tired during the latter part of the day's work and afterwards and had to rest during most of the afternoons. Took some bread in the evening of the 22nd and the morning of the 23rd. Work again on the 24th and 25th. Profuse perspiration but not quite so tired as on the preceding days. Weight on the 25th, 76·5 kg. The technical work on the ergometer was 1·12 Cal. per minute throughout.

A simple inspection of the general table shows:

1. Somewhat irregular variations of the standard metabolism with a distinct tendency to become higher during the last days of the period when the quotient was very low.

2. The correlation between the quotient and the total metabolism during work is marked when the changes from day to day are considered, but it is unmistakable at the same time that the metabolism at a constant quotient becomes gradually lower during the period. We take this lowering to be due to training.

In order to find the most reliable numerical expression for the function studied we have examined and combined the experimental results in several ways.

Table VII.

General Table of Experiments.

	Rest				Work					
Date	Ventilation l. per min.	O_2 per min. cc.	R.Q.	Cal. per min.	Length of period	O_2 per min. cc.	R.Q.	Cal. per min.	Revolut. per min.	Tech. work Cal. per min.
O.H. I				*c*				*b*		*a*
1917	5·93	261	0·82	1·275	30	1399	0·836	6·86	49·4	1·123
8. v.	5·93	258	0·82	1·265	26	1393	0·829	6·83		
					29	1405	0·818	6·87		
10. v.	6·05	290	0·82	1·42	31	1395	0·814	6·82	49·4	1·123
	6·44	292	0·81	1·425	29	1395	0·803	6·81		
					32	1395	0·810	6·82		
11. v.	5·97	303	0·79	1·475	24	1365	0·830	6·69	49·4	1·123
	6·35	296	0·84	1·455	25	1347	0·835	6·61		
					23	1375	0·814	6·72		
14. v.	6·34	276	0·92	1·375	24	1353	0·864	6·68	49·4	1·123
	6·21	258	0·96	1·295	24	1336	0·870	6·60		
					23	1320	0·876	6·53		
15. v.	6·24	291	0·945	1·455	24	1303	0·929	6·50	49·4	1·123
	6·28	279	0·945	1·395	24	1312	0·922	6·54		
					23	1306	0·919	6·51		
17. v.	6·16	280	0·92	1·395	24	1276	0·904	6·34	49·4	1·123
	6·43	272	0·99	1·375	25	1301	0·896	6·45		
					23	1314	0·881	6·50		
18. v.	5·95	276	0·93	1·38	23	1337	0·839	6·56	49·4	1·123
	6·06	286	0·89	1·42	24	1366	0·817	6·68		
					24	1375	0·799	6·70		
21. v.	5·77	300	0·71	1·435	23	1419	0·731	6·82	49·3	1·120
	5·70	307	0·705	1·47	24	1427	0·715	6·85		
					22	1434	0·714	6·88		
22. v.	5·94	291	0·71	1·395	23	1398	0·717	6·71	49·3	1·120
	6·25	291	0·75	1·405	25	1396	0·709	6·70		
					23	1413	0·719	6·78		
24. v.	6·22	324	0·71	1·55	22	1363	0·735	6·56	49·4	1·123
	6·35	317	0·73	1·53	25	1387	0·734	6·63		
					23	1325[1]	0·765[1]	6·42		
25. v.	5·61	292	0·69	1·395	22	1384	0·744	6·68	49·4	1·123
	5·78	304	0·72	1·46	26	1387	0·731	6·68		
					22	1385	0·746	6·68		

[1] The exceptionally large increase in quotient and decrease in metabolism must arouse suspicion but no reason can be found for doubting the technical accuracy of the results.

The determinations of the resting metabolism have been plotted in Fig. 6 as ordinates with the dates as abscissae. The curve drawn shows that the average standard metabolism remained practically constant except during the last few days when the quotients were very low. We observed in the earlier experiments on A. K. and R. E. that with a low quotient the standard metabolism shows a tendency to rise and we may add that this is borne out also by the later series of determinations given below. We have therefore no

hesitation in concluding that the standard metabolism remained constant during the whole period except for the variations correlated with the variations in quotient. The determinations have accordingly been plotted a second time (Fig. 7) with the quotient as abscissa and a curve drawn to represent them.

We have now the material to combine the standard metabolism c with the metabolism during work b in three different ways. We can either:

1. Use the determination of c for each particular day in combination with the determinations of b for the same day.

2. Use the average value for c from all the determinations in combination with each b.

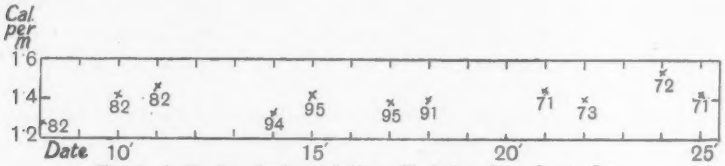

Fig. 6. O. H. Standard metabolism. Variations from day to day. Figures: respiratory quotients.

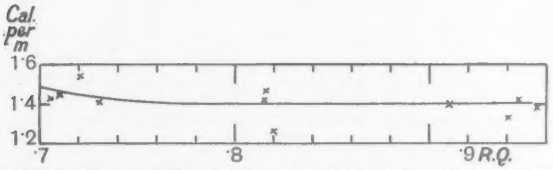

Fig. 7. O. H. Standard metabolism. Variation with respiratory quotient.

3. Use for combination with each b a value for c selected from the curve Fig. 7, on the principle that the respiratory quotient during rest is the same as that during work. Though this latter assumption might seem to be somewhat arbitrary an inspection of Table VII shows unmistakably that there is a very close connection between the quotient during rest and the quotient during work just afterwards, the difference being seldom more than a few per cent.[1]

The alternatives 1 and 2 have been tested in a preliminary calculation of the efficiency at varying quotients the results of which are given in the curves, Fig. 8[2]. When the standard metabolism c found on a particular day depended mainly on some cause which would act also during the work on the same day the combination of the values obtained each day ought to give the most concordant results, but if the variations in the c values are purely accidental and have nothing to do with the results obtained in the corresponding work

[1] This point will be discussed in detail below. Appendix III, p. 354.

[2] In these curves corrections have been introduced for the effect of training according to the principles given below, p. 320.

experiments (which have likewise their own accidental variations) the elimination of the accidental variations of the c values ought to diminish the variability of the final results.

It is seen at a glance that the individual determinations of efficiency show considerably larger deviations from the curve when the c values for each day are utilised than when they are replaced by the average value. A numerical expression for the difference is obtained when the dispersions of the two sets of values are calculated. For Curve I we find a dispersion $\sigma = 0{\cdot}38$ and for Curve II $\sigma = 0{\cdot}31$.

As it might be thought that the correction for the effect of training applied to the curves, Fig. 8, might influence the result of the comparison we have made a similar comparison between the uncorrected values for the expenditure of energy per unit work, using in one case the c values for each day and in the other the c values taken from the curve, Fig. 7, according to the third alternative. The results of this comparison are shown in Fig. 9. It is obvious that the c values taken from the curve make the final results much more uniform than those obtained day by day, and in our opinion this furnishes conclusive evidence in favour of discarding the single determinations and using either the simple average of a number of determinations or a graph in which the accidental variations have been smoothed out[1]. In the final calculations we have also rejected the second alternative (an average c), because it would increase the apparent metabolism per unit work at low quotients, and the later experiments show that there is no reason to doubt the reality of the observed increase in resting metabolism at low quotients.

The curves, Fig. 9, show an increase in e (energy per unit work) when the quotient falls from 0·93 to 0·8, but a slight decrease on the further fall from 0·8 to 0·71. This, as well as certain other irregularities, is due in the main to the fact that the metabolism, when determined at an approximately constant quotient, is evidently decreasing during the 18 days occupied by the series of experiments. In order to examine this decrease we have arranged Table VIII giving for each experimental day the respiratory quotient for the three determinations during work, taken together, the corresponding Calories per minute (b), the metabolism during rest c as found from the curve, Fig. 7, by means of the quotient in column 1, the difference $b-c$, the amount of technical work a and the metabolism per unit work e. The figures in this latter column have been plotted in Fig. 10 as ordinates with the dates as abscissae. All the results corresponding to quotients between 0·78 and 0·88 have been marked by a $\times$ and the others by a small $\bullet$, and it has been attempted to draw a curve representing the variation of the metabolism with time at a quotient of about 0·83. This curve is called the "training curve" and is taken to represent the change in metabolism per unit work depending, as we

[1] We have also in the later series of experiments made similar comparisons between average and individual c values with the same result as that illustrated above, but we do not think it necessary to reproduce them.

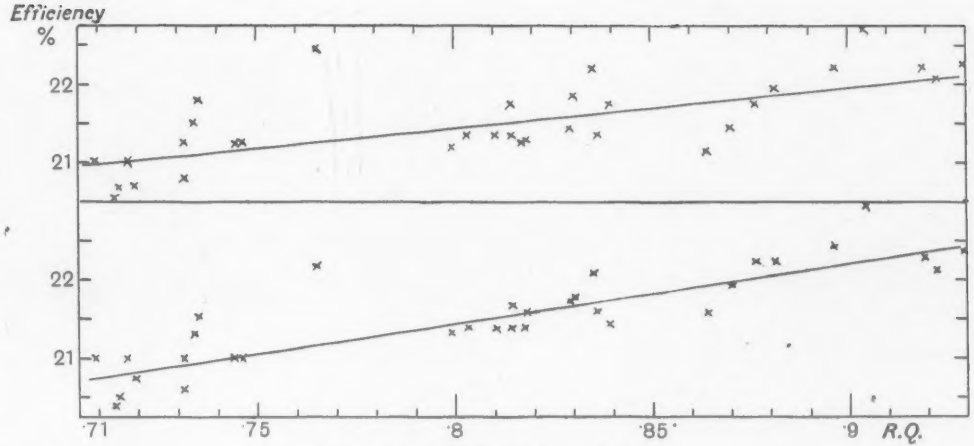

Fig. 8. O. H. Efficiency of muscular work.
Lower curve: average value for standard metabolism. Upper curve: day to day values for standard metabolism.

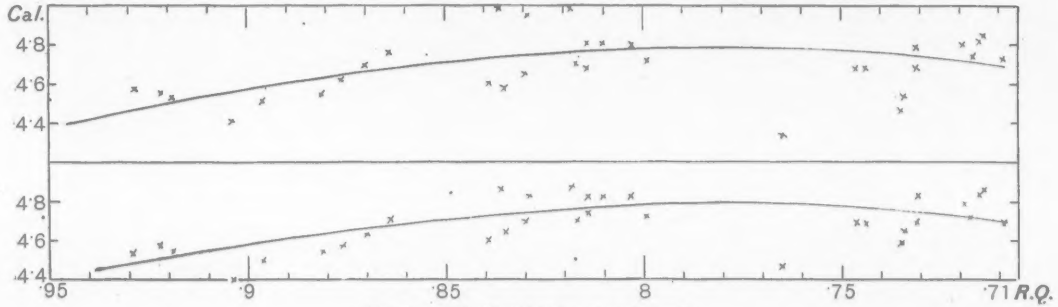

Fig. 9. O. H. Metabolism per unit work.
Lower curve: values for standard metabolism from curve, Fig. 7. Upper curve: day to day values for standard metabolism.

think, upon the increasing training of the subject. Unfortunately we have no determinations at suitable quotients after the 18th and for the last week we have had therefore to extrapolate the training curve as best we might. We have thought it safest to assume a very slight influence of the training after that date.

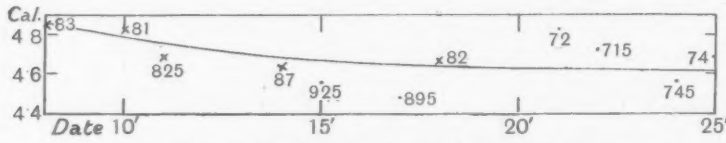

Fig. 10. O. H. Metabolism per unit work. Influence of training.

At the end of the experimental period we assume that the metabolism per unit work at a quotient of 0·83 would have decreased to 4·62 Cal. as shown by the curve, and by finding from the curve the corresponding figure for each experimental day and dividing 4·62 by the same we have obtained a series of figures, increasing from 0·950 on the 8th to 1·000 on the 22nd to 25th, given in the last column of Table VIII as the "correction for training." In the final Table IX the effect of the training has been eliminated by multiplying each e value by the correction coefficient for the day.

Table VIII.

Date 1917	R.Q.	Average Cal. during work b	Cal. at rest from curve c	Difference d	Technical work a	Cal. per unit of work e	Training correction (from Fig. 10)
8. v.	·83	6·85	1·40	5·45	1·123	4·85	·950
10. v.	·81	6·82	1·40	5·42	1·123	4·83	·967
11. v.	·825	6·67	1·40	5·27	1·123	4·69	·973
14. v.	·87	6·60	1·40	5·20	1·123	4·63	·985
15. v.	·925	6·52	1·40	5·12	1·123	4·56	·989
17. v.	·895	6·43	1·40	5·03	1·123	4·48	·993
18. v.	·82	6·65	1·40	5·25	1·123	4·67	·996
21. v.	·72	6·85	1·44	5·41	1·120	4·83	·998
22. v.	·715	6·73	1·45	5·28	1·120	4·72	1·000
24. v.	·745	6·54	1·41	5·13	1·123	4·57	1·000
25. v.	·74	6·68	1·41	5·27	1·123	4·69	1·000

We are fully aware that objections can be raised against this method of attempting an elimination of the training effect. We must admit of course that the training curve as drawn is more or less arbitrary, but we have convinced ourselves that even if the training curve is drawn differently from the one we have adopted in any way compatible with the actual experiments on which it must be based, the resulting effect on the final curve will be very slight.

It must be conceded further that the effect of training is not necessarily the same at all quotients. This point will be touched upon below, p. 334.

Table IX.

Date and number		R.Q.	Cal. work	Cal. obs.	Rest from curve	Difference		Calories per unit work			Deviation from curve
			b	c_{I}	c_{II}	d_{I}	d_{II}	e_{I}	e_{II}	e_{II} corrected	δ
May 1917											
22	2	·709	6·70	1·40	1·45	5·30	5·25	4·73	4·69	4·69	−·10
21	3	·714	6·88	1·45	1·43	5·43	5·45	4·85	4·865	4·855	+·075
21	2	·715	6·85	1·45	1·43	5·40	5·42	4·82	4·84	4·83	+·05
22	1	·717	6·71	1·40	1·42	5·31	5·29	4·74	4·72	4·72	−·055
22	3	·719	6·78	1·40	1·42	5·38	5·36	4·80	4·79	4·79	+·02
21	1	·731	6·82	1·45	1·40	5·37	5·42	4·79	4·84	4·83	+·08
25	2	·731	6·68	1·43	1·40	5·25	5·28	4·68	4·70	4·70	−·05
24	2	·734	6·63	1·54	1·40	5·09	5·23	4·54	4·66	4·66	−·085
24	1	·735	6·56	1·54	1·40	5·02	5·16	4·47	4·60	4·60	−·14
25	1	·744	6·68	1·43	1·40	5·25	5·28	4·68	4·70	4·70	−·03
25	3	·746	6·68	1·43	1·40	5·25	5·28	4·68	4·70	4·70	−·025
24	3	·765	6·42	1·54	1·40	4·88	5·02	4·34	4·47	4·47	−·225
18	3	·799	6·70	1·40	1·40	5·30	5·30	4·72	4·72	4·70	+·06
10	2	·803	6·81	1·42	1·40	5·39	5·41	4·80	4·82	4·66	+·02
10	3	·810	6·82	1·42	1·40	5·40	5·42	4·81	4·825	4·665	+·04
10	1	·814	6·82	1·42	1·40	5·40	5·42	4·81	4·825	4·665	+·05
11	3	·814	6·72	1·47	1·40	5·25	5·32	4·68	4·74	4·61	±·00
18	2	·817	6·68	1·40	1·40	5·28	5·28	4·70	4·70	4·68	+·07
8	3	·818	6·87	1·27	1·40	5·60	5·47	4·99	4·87	4·625	+·015
8	2	·829	6·83	1·27	1·40	5·56	5·43	4·95	4·84	4·60	+·01
11	1	·830	6·69	1·47	1·40	5·22	5·29	4·65	4·71	4·58	−·01
11	2	·835	6·61	1·47	1·40	5·14	5·21	4·58	4·64	4·515	−·065
8	1	·836	6·86	1·27	1·40	5·59	5·46	4·98	4·86	4·615	+·035
18	1	·839	6·56	1·40	1·40	5·16	5·16	4·60	4·60	4·58	±·00
14	1	·864	6·68	1·34	1·40	5·34	5·28	4·76	4·705	4·63	+·095
14	2	·870	6·60	1·34	1·40	5·26	5·20	4·69	4·63	4·56	+·04
14	3	·876	6·53	1·34	1·40	5·19	5·13	4·62	4·57	4·50	−·015
17	3	·881	6·50	1·39	1·40	5·11	5·10	4·55	4·54	4·51	+·005
17	2	·896	6·45	1·39	1·40	5·06	5·05	4·51	4·50	4·47	−·01
17	1	·904	6·34	1·39	1·40	4·95	4·94	4·41	4·40	4·37	−·10
15	3	·919	6·51	1·43	1·40	5·08	5·11	4·53	4·55	4·50	+·055
15	2	·922	6·54	1·43	1·40	5·11	5·14	4·55	4·58	4·53	+·09
15	1	·929	6·50	1·43	1·40	5·13	5·10	4·57	4·54	4·49	+·06
										Sum	−·035

In Table IX all the determinations have been arranged in the order of increasing quotients and in the last column but one the e values are corrected for training by means of the figures given in Table VIII. These corrected e values have been plotted in Fig. 11, which shows very distinctly the regular variation of the expenditure of energy per unit work with the respiratory quotient. It should be noted that the determinations number 3 which are marked o are on the whole slightly above the curve probably on account of fatigue. The determination R.Q. = 0·765 (No. 3, May 24th) forms a very striking exception to this rule and deviates from all the rest in that the third period shows a marked rise in quotient and a great fall in metabolism. As pointed out above (Table VII, note 1) there is no reason to doubt the technical accuracy of this determination.

In the last column of Table IX are given the deviations of the determinations from the curve. Their number is 33 and the dispersion works out as $\sigma = 0{\cdot}072$ Cal. or 1·55 % of the average metabolism $e_{0{\cdot}85} = 4{\cdot}56$ Cal., a very striking improvement on the results of the preliminary series.

The straight line drawn corresponds to an $e_F = 4{\cdot}79$ Cal. and $e_C = 4{\cdot}32$ Cal. The waste of energy from fat is therefore $w = 0{\cdot}47$ Cal. or 9·8 %.

Third Series of Experiments.

The second series of determinations would have been quite satisfactory if the curve of training and the corrections based upon this curve had not been a little uncertain, and we resolved therefore to change the routine so as to insure the best possible data from which to construct a curve of training and at the same time to make the influence of the training as uniform as possible throughout the series. This end we hoped to attain by making determinations each day on the same subject during a period of three weeks and letting the subject undergo repeated changes of diet from carbohydrate to fat and *vice versa* beginning and ending the series on a mixed diet and effecting the changes through the interpolation of one day's mixed diet.

The experiments of this series were carried out in January and February 1918 on Mr A. Möller Nielsen (age 24 years, height 174·5 cm.) a student of languages and gymnastics who kindly undertook to act as a subject and carried out his part of the work very carefully. From his notes which were not so elaborate as those of O. H. we give the following essential points.

Jan. 28th to 30th, ordinary mixed diet, poor in protein. Work on the ergometer from the 29th. Weight on the 29th, 63 kg. No exercise. Carbohydrate diet 31st to Feb. 4th. Mixed diet on the 5th. Went for a short walk (2 km.) in the evening. Fat diet 6th to 9th. A short walk every evening. Felt tired in the evening on the 6th. Mixed diet on the 10th and 11th. Carbohydrate diet 12th and 13th. Fat diet 14th to 16th. Diarrhoea in the afternoon on the 14th. Very tired in the evening. Severe headache on the 15th. Slightly unwell on the 16th. Mixed diet 17th to 19th. Weight on the 19th, 69·8 kg.

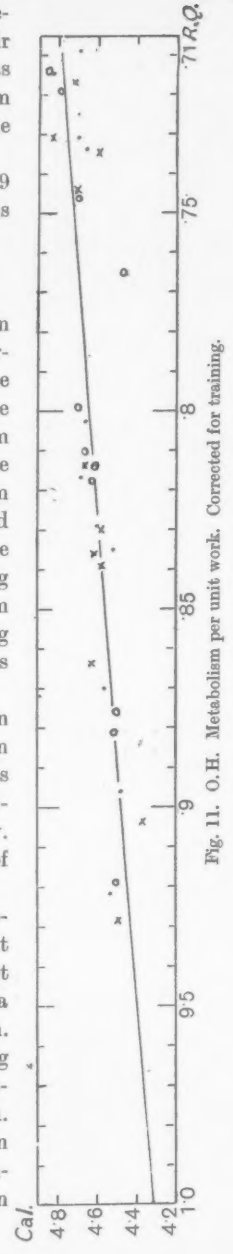

Fig. 11. O.H. Metabolism per unit work. Corrected for training.

Table X.

General Table of Experiments.

	Rest				Work					
Date	Ventilation l. per min.	O_2 per min. cc.	R.Q.	Cal.	Length	O_2 per min. cc.	R.Q.	Cal.	Revolutions per min.	Techn. work Cal.
A. M. N.				*c*				*b*		*a*
1918	6·79	236	·845	1·16	Normal period of work. Training experiment					
29. i.	7·30	226	·855	1·11						
					25	1234	·848	6·07		
30. i.	6·15	231	·82	1·13	21	1080	·873	5·335		
	6·38	212	·84	1·04	22	1075	·862	5·30	49·3	·910
					22	1102	·855	5·43		
31. i.	6·20	221	·87	1·09	20	1050	·890	5·205		
	5·97	213	·87	1·05	20	1057	·888	5·24	49·3	·910
					20	1074	·883	5·32		
1. ii.	6·41	242	·85	1·19	21	1075	·850	5·29		
	6·20	244	·86	1·20	21	1090	·846	5·36	49·3	·910
					21	1113	·846	5·475		
2. ii.	6·00	245	·90	1·215	21	1049	·886	5·20		
	5·60	235	·88	1·165	21	1067	·870	5·27	49·2	·9085
					21	1080	·872	5·335		
3. ii.	6·17	238	·895	1·18	21	1141	·910	5·68		
	6·11	242	·89	1·195	21	1135	·896	5·635	49·2	(1·05[1])
					21	1145	·892	5·68		
4. ii.	6·20	235	·98	1·185	Work as usual but no determinations on account of trouble with ergometer regulator and gas analysis apparatus					
	6·55	242	·99	1·22						
5. ii.	5·61	234	·93	1·175	21	1049	·882	5·19		
	6·01	227	1·01	1·15	21	1039	·907	5·165	49·2	·908
					21	1065	·876	5·265		
6. ii.	5·80	231	·88	1·14	21	1034	·876	5·11		
	5·83	231	·88	1·14	21	1029	·876	5·09	49·2	·908
					21	1054	·868	5·205		
7. ii.	5·00	231	·83	1·13	21	1057	·787	5·14		
	5·07	223	·83	1·095	21	1080	·782	5·25	49·2	·9075
					21	1063	·805	5·19		
8. ii.	5·29	231	·79	1·125	21	1106	·770	5·36		
	5·33	239	·77	1·16	21	1112	·761	5·38	49·2	·9075
					21	(1112)[3]	·764[2]	5·38		
9. ii.	5·17	230	·775	1·115	21	(1060)[3]	(·768)[3]	(5·125)[3]		
	4·81	224	·78	1·085	21	1110	·764	5·375	49·5	·9135
					21	1109	·760	5·365		
10. ii.	5·28	233	·75	1·125	21	1087	·770	5·27		
	5·28	223	·78	1·08	21	1093	·763	5·295	49·5	·9135
					20	1113	·761	5·385		

[1] Regulator on ergometer did not work. The load was determined after the experiment.

[2] Oxygen determination in last average sample lost. Percentage determined by interpolation between remaining samples which were practically constant.

[3] Oxygen determination in first intermediate sample not quite reliable.

Table X *continued.*

	Rest				Work					
Date	Ventilation l. per min.	O_2 per min. cc.	R.Q.	Cal.	Length	O_2 per min. cc.	R.Q.	Cal.	Revolutions per min.	Techn. work Cal.
A. M. N.				*c*				*b*		*a*
11. ii.	5·05	212	·85	1·045	21	1043	·829	5·11		
	5·10	206	·87	1·02	21	1045	·830	5·125	49·5	·9135
					20	1066	·825	5·22		
12. ii.	5·46	217	·94	1·085	20	1000	·889	4·96		
	5·13	214	·90	1·06	21	1000	·884	4·95	49·5	·9135
					21	1026	·876	5·07		
13. ii.	6·16	216	·97	1·085	21	988	·930	4·935		
	6·19	219	·99	1·105	21	995	·925	4·965	49·5	·9135
					20	1020	·928	5·09		
14. ii.	6·23	214	1·06	1·095	21	(1030)[1]	(·926)[1]	(5·14)[1]		
	6·00	212	1·03	1·075	20	1023	·924	5·10	49·5	·9135
					20	1030	·940	5·15		
15. ii.	5·30	216	·83	1·06	21	1016	·841	4·99		
	4·81	205	·84	1·005	21	1031	·838	5·06	49·5	·9135
					21	1040	·842	5·11		
16. ii.	5·16	221	·79	1·075	21	1063	·793	5·16		
	4·94	212	·81	1·035	21	1089	·779	5·275	49·4	·912
					21	1052	·802	5·13		
17. ii.	5·10	214	·81	1·045						
	5·38	213	·79	1·035	70	1120	·719	5·375	49·4	·912
18. ii.	5·56	208	·97	1·045	22	(998)[2]	(·843)[2]	(4·91)[2]		
	5·35	204	·92	1·02	21	1011	·829	4·96	49·4	·9115
					21	1033	·833	5·065		
19. ii.	4·98	206	·86	1·015	21	1007	·844	4·95		
	5·10	209	·85	1·03	21	1020	·846	5·015	49·4	·9115
					21	1022	·840	5·02		

[1] The oxygen determination in the first average sample is probably erroneous. The first intermediate sample gave an O_2 deficit of 0·493 % the second gave 0·4935, while the intervening average sample gave 0·5085 %. The probable values would be oxygen per min. 1004 cc., R.Q. 0·95, calories 5·025.

[2] First average sample lost. Values obtained by interpolation between intermediate samples.

During each day's work on the ergometer this subject noticed no distinct difference between the effects of the different diets and never became very tired. The technical work per minute was 0·91 Cal.

The determinations of the standard metabolism show a very marked decline during the whole of the experimental period, a decline which is brought out very clearly when the results are arranged graphically with the dates as abscissae (Fig. 12). The three first determinations give low values, but otherwise there is a steady and extremely regular decrease from first to last. The low values observed on the three first days are probably due to a fall in the body temperature. When the subject suppressed all movements, as he did very carefully throughout, he complained of feeling cold. In all the later determinations he was carefully wrapped up and felt warm. The decrease in standard metabolism, taking place at all events from Feb. 1st to 19th, is

remarkable, and the more so as the subject gained in weight from 63 to 70 kg. As the diet during the experimental period was poor in protein it is conceivable that the decrease may be due to the elimination of a nitrogen reserve from the body as in the recent experiments of Benedict and his collaborators [1918], but we have no observations to support such a hypothesis.

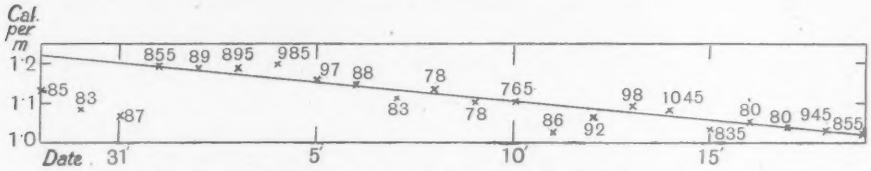

Fig. 12. A. M. N. Standard metabolism. Variation from day to day.

As the quotient during rest has in all experiments been above 0·77 there is very little influence of the quotient upon the resting metabolism to be detected, and we have therefore used the values for standard metabolism deduced from the straight line curve, Fig. 12, in the subsequent treatment of the material.

Table XI.

Date 1918	R.Q. work	Average Cal. during work *b*	Cal. at rest from curve *c*	Difference *d*	Technical work *a*	Cal. per unit work *e*	Training correction (from Fig. 13)
30. i.	·865	5·355	1·21	4·145	·910	4·555	·952
31. i.	·885	5·255	1·20	4·055	·910	4·46	·957
1. ii.	·85	5·375	1·19	4·185	·910	4·60	·962
2. ii.	·875	5·27	1·18	4·09	·9085	4·50	·969
3. ii.	·90	5·665	1·17	4·495	(1·05)[1]	(4·35)[1]	·973
5. ii.	·89	5·205	1·15	4·055	·908	4·47	·982
6. ii.	·875	5·135	1·14	3·995	·908	4·40	·985
7. ii.	·79	5·19	1·13	4·06	·9075	4·47	·989
8. ii.	·765	5·37	1·12	4·25	·9075	4·685	·991
9. ii.	·76	5·36	1·115	4·245	·9135	4·65	·993
10. ii.	·765	5·32	1·105	4·215	·9135	4·615	·995
11. ii.	·83	5·15	1·095	4·055	·9135	4·44	·997
12. ii.	·885	4·995	1·085	3·91	·9135	4·28	·999
13. ii.	·93	5·00	1·075	3·925	·9135	4·30	1·000
14. ii.	·93	5·13	1·07	4·06	·9135	4·445	1·000
15. ii.	·84	5·055	1·06	3·995	·9135	4·375	1·000
16. ii.	·79	5·19	1·05	4·14	·912	4·54	1·001
17. ii.	·72	5·375	1·04	4·335	·912	4·755	1·002
18. ii.	·83	5·00	1·03	3·97	·9115	4·355	1·002
19. ii.	·845	4·995	1·02	3·975	·9115	4·36	1·002

[1] Doubtful.

In Table XI the determinations during work done on each day have been averaged and the metabolism per unit work calculated for each separate day taken as a whole, and in Fig. 13 these results have been plotted with the dates as abscissae. By means of the values showing quotients between 0·82

and 0·89 a training curve has been constructed. The distribution of the determinations on which this curve is based shows that its possible errors must be comparatively slight. Owing to the distribution of the fat and carbohydrate periods the difference between the efficiency of these diets for work comes out about as distinctly and practically the same quantitatively with no correction whatever for training. See Figs. 14 and 15.

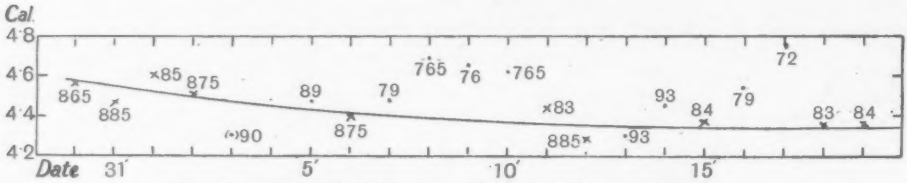

Fig. 13. A. M. N. Metabolism per unit work. Influence of training.

In the final Tables XII–XIII the determinations are arranged as usual according to their quotients, and as an inspection of the general Table X shows that the metabolism in the third experimental periods is generally a little higher than in periods 1 and 2, the third periods have been sorted out and arranged in the separate Table XIII. The results have been plotted and curves drawn to represent them in Figs. 14 and 15. In drawing the curves we have *not* attempted to represent all the determinations as nearly as possible. That would obviously mean curves which were not straight lines but slightly convex towards the abscissa, but such curves would be incompatible with our theoretical conception.

We can safely maintain, however, that the lines, drawn straight to be in accordance with theory, are perfectly compatible with the determinations, since the dispersion of these is only $\sigma = 0{\cdot}074$ Cal. or 1·7 % for the curve representing periods 1 and 2 and $\sigma = 0{\cdot}080$ Cal. or 1·8 % for the curve representing period 3.

It should be pointed out that the comparatively large deviation of a few determinations *upwards* from the curve as at R.Q. = 0·924 in Fig. 14 and at 0·940 in Fig. 15 is a thing which must be expected in experiments of this kind. A slight indisposition on the part of the subject will be enough to account for them.

The straight line, Fig. 14, representing periods 1 and 2 corresponds to an $e_F = 4{\cdot}70$ Cal. and $e_C = 4{\cdot}02$. The waste of energy from fat is therefore 0·68 Cal. or 14·5 %. The line in Fig. 15 representing the third experimental periods corresponds to $e_F = 4{\cdot}74$ Cal. and $e_C = 4{\cdot}11$ Cal. or a waste of energy from fat of 0·63 Cal. = 13·3 %.

Fourth Series of Experiments.

Our fourth and final series of determinations was again made on the subject O. H. on whom we had obtained in 1917 the remarkably constant results given above pp. 314—322. Mr H. had been working hard for his

degree during the preceding term and his general physical training was therefore below his usual standard. During a fortnight preceding the experiments he undertook to make a bicycle ride of about 30 km. every day. We hoped thereby to induce such an amount of bicycle training that the training effect of the experiments themselves, which had been so pronounced in the two preceding series, would be greatly diminished. As will be seen below this hope was in the main disappointed. In all other respects the routine was the same as that adopted in the third series.

Table XII.

Date and number Jan. and Feb. 1918		R.Q. work	Work Cal. *b*	Rest Cal. *c*	Difference *d*	Cal. per unit work *e*	Cal. per unit work *e* corrected	Deviation from curve *δ*
8	2	·761	5·38	1·12	4·26	4·69	4·645	+·065
10	2	·763	5·295	1·105	4·19	4·59	4·57	±·00
9	2	·764	5·375	1·115	4·26	4·665	4·635	+·065
9	1	(·768)	(5·125)	1·115	(4·01)	(4·39	4·36)[1]	
8	1	·770	5·36	1·12	4·24	4·67	4·625	+·065
10	1	·770	5·27	1·105	4·165	4·56	4·54	−·02
16	2	·779	5·275	1·05	4·225	4·63	4·635	+·095
7	2	·782	5·25	1·13	4·12	4·54	4·49	−·04
7	1	·787	5·14	1·13	4·01	4·42	4·37	−·15
16	1	·793	5·16	1·05	4·11	4·51	4·515	+·01
11	1	·829	5·11	1·095	4·015	4·395	4·38	−·04
18	2	·829	4·96	1·03	3·93	4·31	4·32	−·10
11	2	·830	5·125	1·095	4·03	4·41	4·40	−·02
15	2	·838	5·06	1·06	4·00	4·38	4·38	−·02
15	1	·841	4·99	1·06	3·93	4·305	4·305	−·085
18	1	·843	(4·91)	1·03	(3·88)	(4·26	4·27)[2]	
19	1	·844	4·95	1·02	3·93	4·31	4·32	−·06
1	2	·846	5·36	1·19	4·17	4·585	4·41	+·03
19	2	·846	5·015	1·02	3·995	4·385	4·395	+·15
1	1	·850	5·29	1·19	4·10	4·51	4·34	−·03
30	2	·862	5·30	1·21	4·09	4·495	4·28	−·06
2	2	·870	5·27	1·18	4·09	4·50	4·36	+·04
30	1	·873	5·335	1·21	4·125	4·535	4·32	±·00
6	1	·876	5·11	1·14	3·97	4·375	4·31	±·00
6	2	·876	5·09	1·14	3·95	4·35	4·285	−·025
5	1	·882	5·19	1·15	4·04	4·45	4·37	+·075
12	2	·884	4·95	1·085	3·865	4·23	4·225	−·065
2	1	·886	5·20	1·18	4·02	4·425	4·29	+·005
31	2	·888	5·24	1·20	4·04	4·44	4·25	−·03
12	1	·889	4·96	1·085	3·875	4·24	4·235	−·045
31	1	·890	5·205	1·20	4·005	4·40	4·21	−·07
3	2	·896	5·635	1·17	4·46	(4·3	4·2)[3]	
5	2	·907	5·165	1·15	4·015	4·42	4·34	+·10
3	1	·910	5·68	1·17	4·51	(4·3	4·2)[3]	
14	2	·924	5·10	1·07	4·03	4·41	4·41	+·21
13	2	·925	4·965	1·075	3·89	4·26	4·26	+·065
14	1	(·926)	5·14	1·07	4·07	(4·455	4·455)[4]	
13	1	·930	4·935	1·075	3·86	4·225	4·225	+·04
							Sum	+·185

[1] Slightly uncertain. [2] Slightly uncertain. [3] Doubtful. [4] Doubtful.

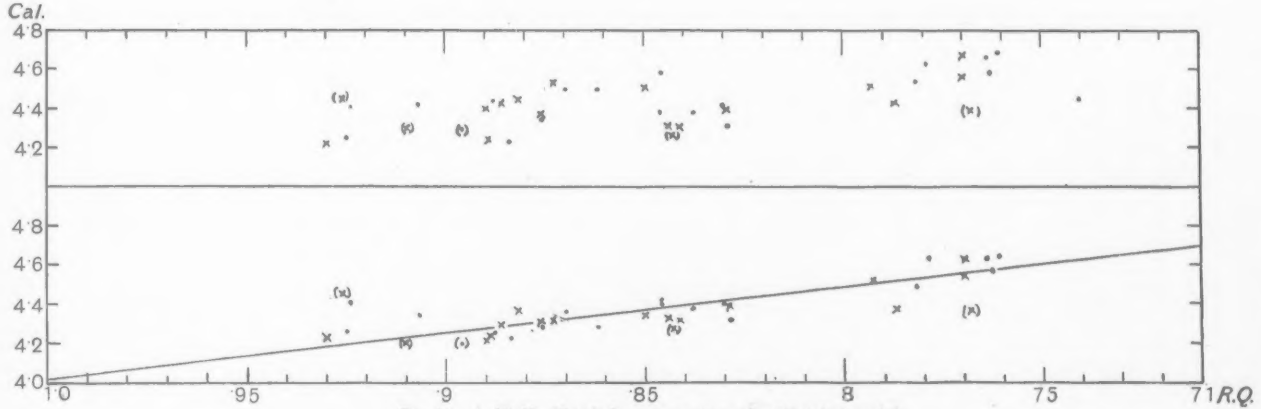

Fig. 14. A. M. N. Metabolism per unit work. Periods 1 and 2.
Lower section: values corrected for training. Upper section: values not corrected for training.

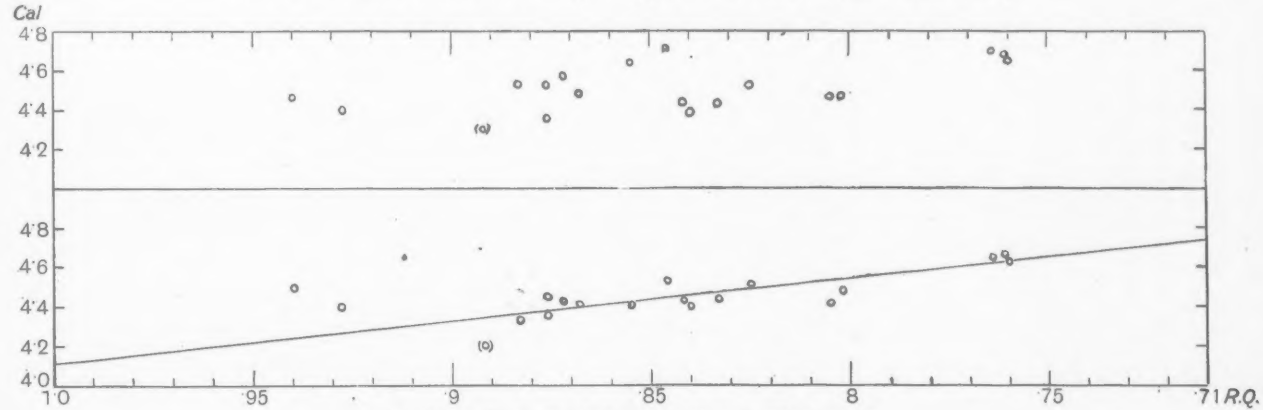

Fig. 15. A. M. N. Metabolism per unit work. Third periods.
Lower section: values corrected for training. Upper section: values not corrected for training.

Table XIII.

9	3	·760	5·365	1·115	4·25	4·655	4·625	−·01
10	3	·761	5·385	1·105	4·28	4·685	4·665	+·03
8	3	·764	5·38	1·12	4·26	4·695	4·65	+·03
16	3	·802	5·13	1·05	4·08	4·475	4·48	−·06
7	3	·805	5·19	1·13	4·06	4·47	4·42	−·11
11	3	·825	5·22	1·095	4·125	4·52	4·51	+·02
18	3	·833	5·065	1·03	4·035	4·43	4·44	−·03
19	3	·840	5·02	1·02	4·00	4·39	4·40	−·055
15	3	·842	5·11	1·06	4·05	4·435	4·435	−·015
1	3	·846	5·475	1·19	4·285	4·71	4·53	+·085
30	3	·855	5·43	1·21	4·22	4·64	4·41	−·01
6	3	·868	5·205	1·14	4·065	4·48	4·41	+·015
2	3	·872	5·335	1·18	4·155	4·575	4·43	+·04
5	3	·876	5·265	1·15	4·115	4·53	4·45	+·07
12	3	·876	5·07	1·085	3·985	4·365	4·36	−·015
31	3	·883	5·32	1·20	4·12	4·53	4·335	−·025
3	3	·892	5·68	1·17	4·51	(4·3	4·2)[1]	
13	3	·928	5·09	1·075	4·015	4·40	4·40	+·135
14	3	·940	5·15	1·07	4·08	4·47	4·47	+·23
							Sum	+·145

[1] Doubtful.

From the notes of the subject we give the following extract:

When the experiments began on April 2nd the general athletic training was distinctly lower than in 1917 but the special training for bicycling was better. The fatigue *during* work on the ergometer did not become very pronounced, but in the afternoon the subject was almost constantly tired and had to take rest on a couch for 1½ to 2 hours. The difference between the fatigue on fat days and carbohydrate days was less pronounced than in 1917 but on several occasions very distinct, nevertheless. Work was performed on the ergometer each day throughout the period and no other exercise was taken except in a few instances specified below.

Mixed diet from 2nd to 5th. Somewhat tired in the afternoon and evening. Weight on the 4th, 84·3 kg.

Fat diet, 6th to 8th. Not distinctly fatigued during the riding, but repeatedly suspected the metronome to be too fast, though as a matter of fact it was rather slower than the normal rate of pedalling of the subject. Somewhat tired during each afternoon and evening.

Mixed diet on the 9th. Weight, 81·5 kg.

Carbohydrate diet, 10th to 13th. On the 11th less tired than usual, took no rest in the afternoon. 12th tired for a short period during the work, tired in the afternoon with feeling of "pressure" in the abdomen. No objective symptoms of digestive trouble. 13th, symptoms and fatigue less pronounced. Weight, 83·5 kg. 14th, felt well during the work. No abdominal "pressure." Very slight fatigue afterwards.

Mixed diet, 14th to 17th. 15th, 18th, again rather tired in the afternoon. Weight on the 15th, 83·1 kg., on the 18th 82·2 kg.

Table XIV.

General Table of Experiments.

	Rest				Work					
Date	Ventilation l. per min.	O_2 per min. cc.	R.Q.	Cal.	Length of per.	O_2 per min. cc.	R.Q.	Cal.	Revolutions per min.	Techn. work Cal.
O.H. II				*c*				*b*		*a*
1918	5·71	263	·79	1·28	21	1304	·855	6·42		
3. iv.	(6·50	260	·86)[1]	1·28	21	1317[2]	·843	6·475	49·3	1·122
					21	1316[2]	·845	6·47		
4. iv.	5·95	275	·815	1·345	21	1347	·840	6·62		
	5·32	242	·80	1·18	21	1334	·839	6·555	49·5	1·127
					21	1327	·831	6·508		
5. iv.	5·63	269	·84	1·32	20	1290	·858	6·36		
	5·39	266	·815	1·30	20	1284	·846	6·315	49·4	1·125
					20	1321	·826	6·47		
6. iv.	5·52	263	·785	1·28	20	1300	·819	6·36		
	5·505	256	·795	1·25	20	1275	·839	6·26	49·3	1·122
					20	1300	·830	6·375		
7. iv.	5·58	288	·75	1·39	20	1339	·768	6·49		
					20	1345	·766	6·52	49·4	1·125
					20	1355	·763	6·56		
8. iv.	5·13	282	·695	1·35	21	1381	·728	6·64		
	5·67	285	·725	1·38	21	1361	·740	6·56	49·5	1·128
					21	1386	·732	6·67		
9. iv.	5·38	314	·645	(1·42)[3]	21	1333	·751	6·44		
	5·20	292	·66		21	1366	·737	6·58	49·5	1·128
					21	1348	·748	6·51		
10. iv.	5·75	254	·83	1·245	21	1333	·797	6·50		
	5·64	252	·84	1·24	21	1320	·804	6·44	49·5	1·130
					21	1347	·804	6·57		
11. iv.	6·23	248	·985	1·25	21	1270	·904	6·315		
	6·38	252	·995	1·275	21	1271	·903	6·32	49·5	1·128
					21	1280	·902	6·36		
12. iv.	6·47	255	1·00	1·29	21	1244	·944	6·225		
	6·33	260	·97	1·31	21	1231	·945	6·16	49·4	1·126
					21	1261	·917	6·28		
13. iv.	6·49	248	·985	1·25	21	1227	·961	6·16		
	6·63	266	·94	1·33	21	1217	·967	6·12	49·4	1·127
					21	1250	·944	6·26		
14. iv.	6·55	244	1·01	1·25	21	1211	·974	6·10		
	7·05	274	1·00	1·385	21	1255	·938	6·275	49·5	1·128
					21·	1236	·940	6·185		
15. iv.	6·36	260	·845	1·275	21	1221	·904	6·07		
	6·19	263	·855	1·295	26	1235	·895	6·13	49·4	1·126
					21	1250	·882	6·185		

[1] The high quotient certainly due to over-ventilation.

[2] Oxygen determination in intermediate sample 3 obviously erroneous. Value obtained by interpolation.

[3] Caloric value of oxygen by extrapolation in Zuntz's table.

Table XIV *continued.*

	Rest				Work					
Date	Ventilation l. per min.	O_2 per min. cc.	R.Q.	Cal.	Length of per.	O_2 per min. cc.	R.Q.	Cal.	Revolutions per min.	Techn. work Cal.
O.H. II				*c*				*b*		*a*
1918	5·78	256	·83	1·255	21	1254	·862	6·18		
16. iv.	5·80	262	·805	1·275	21	1268	·851	6·24	49·5	1·129
					21	1269	·849	6·24		
17. iv.	5·90	260	·79	1·265	21	1282	·816	6·27		
	5·96	277	·80	1·35	21	1289	·811	6·30	49·4	1·126
					21	1338	·791	6·51		
18. iv.	5·52	261	·77	1·265	21	1285	·808	6·275[1]		
	5·81	261	·79	1·27	21	1287	·808	6·28	49·5	1·127
					21	1310	·800	6·39		
19. iv.	5·81	248	·905	1·23	21	1256	·880	6·22		
	5·67	245	·885	1·215	21	1248	·884	6·18	49·4	1·126
					21	1264	·870	6·24		
20. iv.	6·54	266	·94	1·33	21	(1183	·966	5·945)[2]		
	6·43	261	·95	1·31	21	1225	·935	6·12	49·4	1·127
					21	1236	·937	6·18		
21. iv.	6·43	248	·98	1·25	21	1209	·950	6·055		
	6·52	261	·98	1·315	21	1204	·939	6·02	49·4	1·124
					21	1192	·970	6·00		
22. iv.	6·15	201	1·13		21	1192	·951	5·98		
	6·81	262	·97	1·32	21	1192	·935	5·96	49·4	1·126
					21	1210	·949	6·06		
23. iv.	5·32	213	·84	1·045	21	1255	·796	6·11		
	5·64	223	·87	1·10	21	1276	·788	6·21	49·5	1·128
					21	1295	·778	6·29		
24. iv.	6·46	275	·73	1·32	21					
	5·91	264	·725	1·27	21	(1332	·752	6·44)[3]	49·3	1·124
					21	1334	·760	6·455		
25. iv.	6·59	258	·855	1·27	21	1252	·832	6·14		
	6·35	244	·88	1·205	21	1260[4]	·822	6·17	49·4	1·124
					21	1264[4]	·829	6·20		
26. iv.	6·03	254	·875	1·255	21	1249	·838	6·13		
	5·59	245	·825	1·20	20	1255	·846	6·17	49·4	1·124
					21	1247	·848	6·135		
27. iv.	5·84	240	·86	1·185	21	1227	·890	6·08		
	6·05	254	·87	1·255	18	1212	·907	6·03	49·3	1·124
					21	1233	·892	6·11		
									Average:	1·126

[1] All the average samples lost. Experiments calculated from intermediate samples which varied little and regularly.

[2] Oxygen determination in first average sample somewhat improbable: O_2 deficit in sample 1 0·645 %, sample A 0·6175 %, sample 2 0·625 %.

[3] Intermediate samples 1 and 2 lost. 2 obtained by interpolation.

[4] Intermediate sample 3 lost. Value obtained by interpolation.

Carbohydrate diet, 18th to 21st. Distinctly less tired than before but more so than on the corresponding diet in 1917. On the 21st went for a walk of 10 km. (Had not felt any inclination to take exercise during the preceding days of the experimental period.) Weight on the 22nd, 83·1 kg.

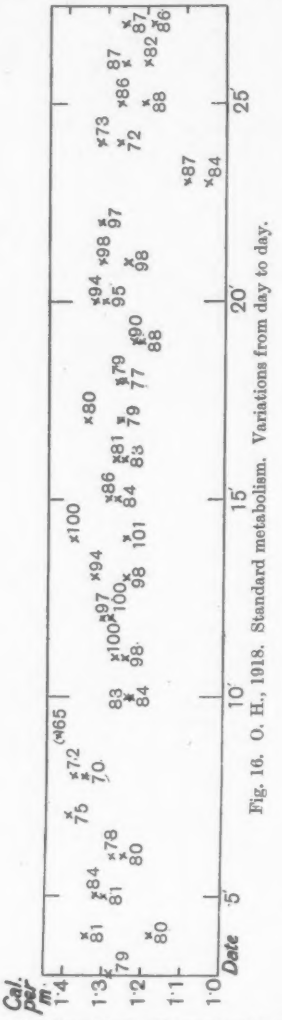

Fig. 16. O. H., 1918. Standard metabolism. Variations from day to day.

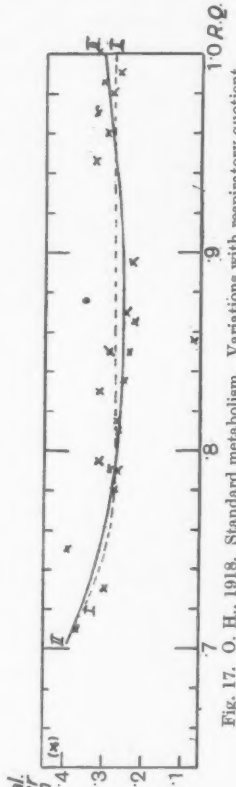

Fig. 17. O. H., 1918. Standard metabolism. Variations with respiratory quotient.

Fat diet, 22nd to 23rd. Very tired in the evening of the 22nd. Tired during the work on the 23rd. Very tired and hungry in the afternoon and evening. Work on the 24th very difficult to perform though somewhat easier during the last half hour. Weight on the 24th, 81·3 kg.

Mixed diet, 24th to 26th. Distinctly less tired after and especially during the work on the 25th to 27th. Weight on the 27th, 82·8 kg.

In Fig. 16 the single experiments made during rest have been plotted with the dates as abscissae and it is seen that while the standard metabolism does not vary with the time the determinations showing a low quotient are distinctly higher than the rest. In Fig. 17 the results (averages for each day) have therefore been plotted again with the respiratory quotient as abscissa. The standard metabolism appears to be distinctly lower at intermediate quotients than it is at low or very high ones. The full line curve drawn represents, we think, fairly accurately all the results except the single one at 0·855. As the increase in metabolism at high quotients might possibly be due to accidental errors we have drawn also the dotted line curve shown, and all the succeeding calculations of the metabolism per unit work have been carried out with both sets of values for the resting metabolism. A discussion of the influence of the quotient upon the standard metabolism will be given below in Appendix II dealing with all the determinations made on the various subjects.

Table XV.

Date	R.Q. work	Average Cal. during work b	Cal. at rest from Curve I c_I	Cal. at rest from Curve II c_{II}	Difference d_I	Difference d_{II}	Cal. per unit work e_I	Cal. per unit work e_{II}	Training correction (from Fig. 18)
3. iv.	·85	6·455	1·27	1·25	5·185	5·205	4·605	4·62	·937
4. iv.	·835	6·56	1·27	1·25	5·29	5·31	4·70	4·715	·941
5. iv.	·845	6·38	1·27	1·25	5·11	5·13	5·54	4·56	·945
6 iv.	·83	6·33	1·27	1·25	5·06	5·08	4·495	4·515	·949
7. iv.	·765	6·52	1·27	1·29	5·25	5·23	4·66	4·645	·952
8. iv.	·735	6·62	1·31	1·33	5·31	5·29	4·72	4·70	·955
9. iv.	·745	6·51	1·295	1·31	5·215	5·20	4·63	4·62	·958
10. iv.	·80	6·50	1·27	1·26	5·23	5·24	4·645	4·655	·962
11. iv.	·905	6·33	1·27	1·26	5·06	5·07	4·495	4·505	·964
12. iv.	·935	6·22	1·27	1·27	4·95	4·95	4·395	4·395	·968
13. iv.	·955	6·18	1·27	1·275	4·91	4·905	4·36	4·36	·971
14. iv.	·95	6·19	1·27	1·27	4·92	4·92	4·37	4·37	·973
15. iv.	·895	6·13	1·27	1·255	4·86	4·875	4·32	4·33	·976
16. iv.	·855	6·22	1·27	1·25	4·95	4·97	4·395	4·415	·979
17. iv.	·805	6·36	1·27	1·26	5·09	5·10	4·52	4·53	·981
18. iv.	·805	6·315	1·27	1·26	5·045	5·055	4·48	4·49	·983
19. iv.	·88	6·21	1·27	1·25	4·94	4·96	4·39	4·405	·985
20. iv.	·935	6·15	1·27	1·265	4·88	4·885	4·335	4·34	·987
21. iv.	·95	6·025	1·27	1·27	4·755	4·755	4·225	4·225	·989
22. iv.	·945	6·00	1·27	1·27	4·73	4·73	4·20	4·20	·991
23. iv.	·79	6·20	1·27	1·27	4·93	4·93	4·38	4·38	·993
24. iv.	·76	6·455	1·28	1·295	5·175	5·16	4·595	4·585	·995
25. iv.	·83	6·17	1·27	1·25	4·90	4·92	4·35	4·37	·9965
26. iv.	·845	6·145	1·27	1·25	4·875	4·895	4·33	4·35	·998
27. iv.	·895	6·07	1·27	1·255	4·80	4·815	4·265	4·28	·999

In Table XV the determinations are averaged for each day and arranged for the construction of a training curve which is given in Fig. 18. As only the experiments showing quotients between 0·8 and 0·9 are utilised for the con-

struction of the curve the difference between the two sets of values for e will be the same throughout and we have therefore utilised the second set only for drawing the curve. A training curve corresponding to e_I would be parallel to the one drawn but lie 0·02 Cal. lower. It is evident that training curves representing the determinations with quotients from 0·73 to 0·8 or with quotients between 0·9 and 0·96 would be very nearly parallel to the curve drawn in Fig. 18, and it follows therefore that the training effect is substantially the same at different quotients—as it ought to be theoretically.

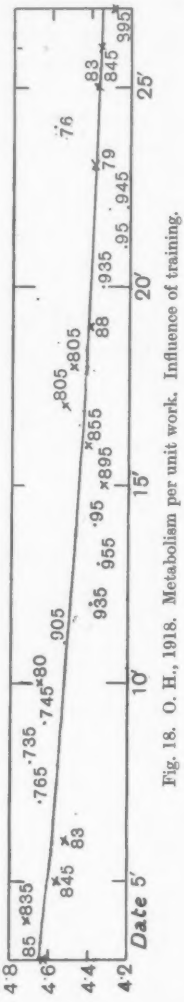

Fig. 18. O. H., 1918. Metabolism per unit work. Influence of training.

It is worthy of note that the training effect of the experiments is considerable and persistent in spite of the measures taken to reduce it (the fortnight's preliminary training). There is no approach to a constant level, and the only advantage we have gained is that we have avoided the steep initial part of the training curves, Figs. 10 and 13, which makes the correction of the first experiments somewhat uncertain.

In the final Tables XVI and XVII we have again sorted out the third periods which show a slightly higher metabolism than the first and second. We have made the calculations with the two sets of values for the standard metabolism c_I and c_{II} obtained from curves I and II, Fig. 17 and corrected them for training by means of the figures given in Table XV. Both sets of corrected values and one set of the uncorrected have been plotted in Figs. 19 and 20 and curves drawn to represent them.

The lower curves in Fig. 19 and Fig. 20, corresponding to the standard metabolism curve II, Fig. 17, which gave the most accurate representation of the rest experiments and showed an increase in metabolism both at very high and at low quotients, are practically straight lines while the best curves (middle curves Fig. 19 and Fig. 20) corresponding to the values e_I are slightly convex towards the abscissa. We believe that the straight line curves are the most reliable.

The curves representing the results e_{II} as uncorrected for training are likewise straight and show about the same waste of energy from fat as do the corrected curves. This is due of course to the experiments with high and low quotients being suitably distributed. Had the experiments with low quotients been all made first the apparent waste would have become greater, and it could have been almost abolished by making the experiments at

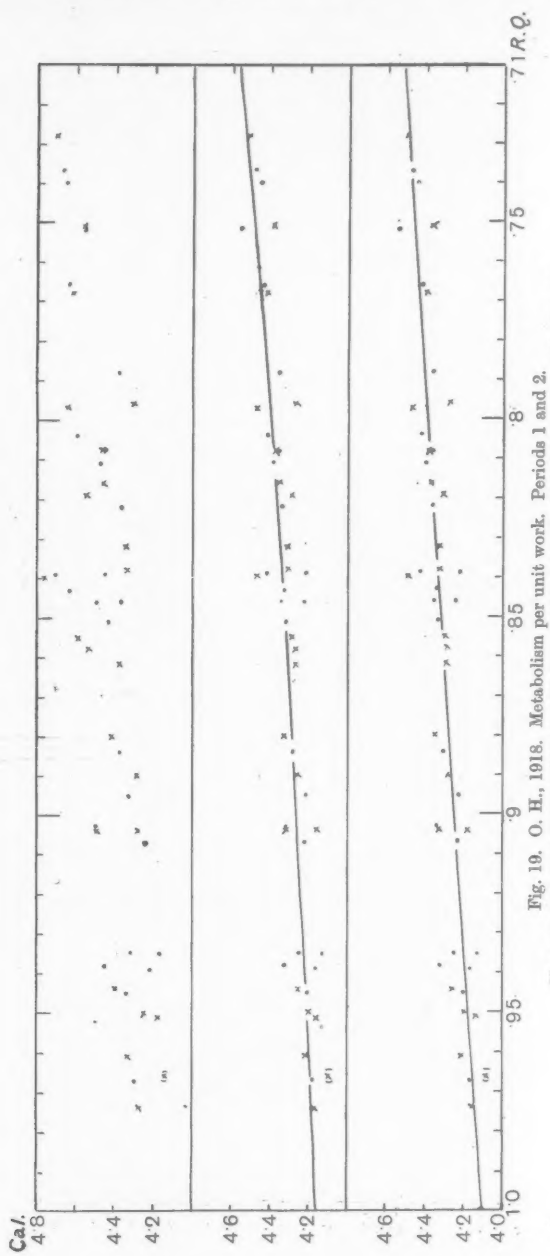

Fig. 19. O. H., 1918. Metabolism per unit work. Periods 1 and 2.
Upper curve: not corrected for training. Standard metabolism from curve II, Fig. 17.
Middle curve: corrected for training. Standard metabolism from curve I, Fig. 17.
Lower curve: corrected for training. Standard metabolism from curve II, Fig. 17.
The middle curve has been drawn about 0·02 Cal. too high.

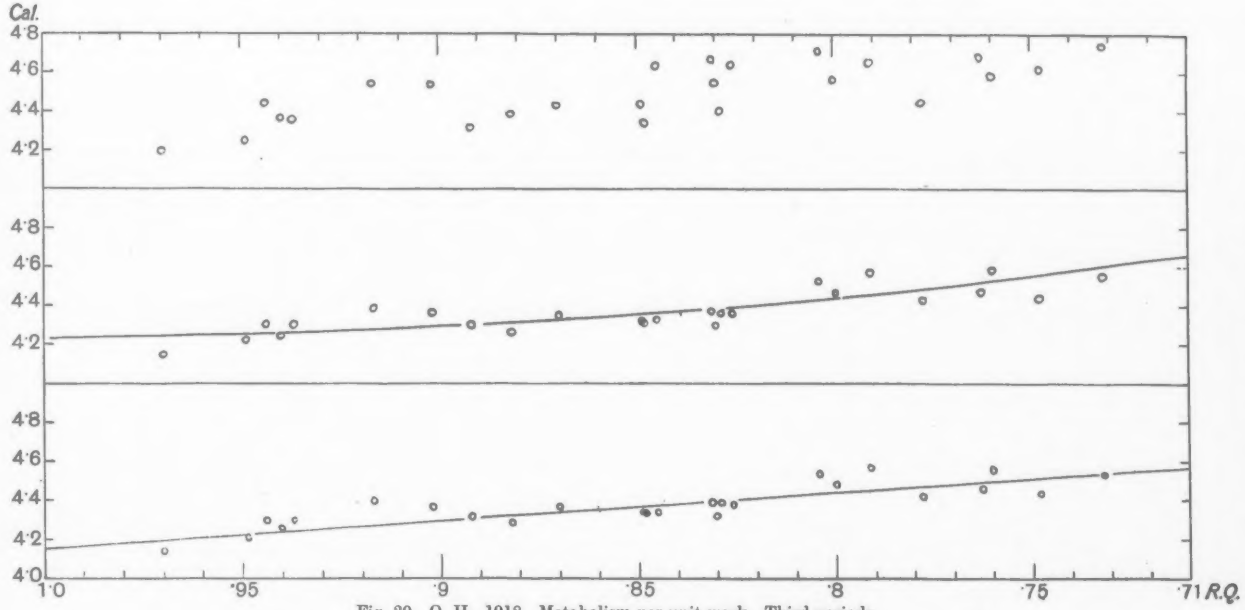

Fig. 20. O. H., 1918. Metabolism per unit work. Third periods.
Upper curve: not corrected for training. Standard metabolism from curve II, Fig. 17.
Middle curve: corrected for training. Standard metabolism from curve I, Fig. 17.
Lower curve: corrected for training. Standard metabolism from curve II, Fig. 17.

Table XVI.

Date and number April 1918	R.Q. work	Work Cal. b	Rest c_I	Cal. c_{II}	Difference d_I	Difference d_{II}	Cal. per unit work e_I	e_{II}	e_I corr.	e_{II} corr.	Deviation from curve (e_{II} corr.) δ
8. 1	·728	6·64	1·32	1·34	5·32	5·30	4·725	4·71	4·51	4·50	+·01
9. 2	·737	6·58	1·305	1·325	5·275	5·255	4·685	4·67	4·49	4·475	±·00
8. 2	·740	6·56	1·30	1·32	5·26	5·24	4·67	4·655	4·46	4·45	−·03
9. 1	·751	6·44	1·29	1·305	5·15	5·135	4·575	4·56	4·38	4·37	−·09
24. 2	·752	6·44	1·29	1·305	5·15	5·135	4·575	4·56	4·55	4·54[1]	+·08
7. 2	·766	6·52	1·27	1·29	5·25	5·23	4·66	4·645	4·44	4·42	−·02
7. 1	·768	6·49	1·27	1·29	5·22	5·20	4·64	4·62	4·42	4·40	−·04
23. 2	·788	6·21	1·27	1·27	4·94	4·94	4·39	4·39	4·36	4·36	−·045
23. 1	·796	6·11	1·27	1·265	4·84	4·845	4·30	4·305	4·27	4·275	−·125
10. 1	·797	6·50	1·27	1·265	5·23	5·235	4·645	4·65	4·47	4·47	+·075
10. 2	·804	6·44	1·27	1·26	5·17	5·18	4·59	4·60	4·42	4·43	+·04
18. 1	·808	6·275	1·27	1·255	5·005	5·02	4·445	4·46	4·37	4·38[2]	±·00
18. 2	·808	6·28	1·27	1·255	5·01	5·025	4·45	4·465	4·37	4·39[2]	+·01
17. 2	·811	6·30	1·27	1·255	5·03	5·045	4·47	4·48	4·39	4·40	+·02
17. 1	·816	6·27	1·27	1·25	5·00	5·02	4·44	4·46	4·36	4·38	+·01
6. 1	·819	6·36	1·27	1·25	5·09	5·11	4·52	4·54	4·29	4·31	−·055
25. 2	·822	6·17	1·27	1·25	4·90	4·92	4·355	4·37	4·34	4·36	±·00
25. 1	·832	6·14	1·27	1·25	4·87	4·89	4·325	4·345	4·31	4·33	−·01
26. 1	·838	6·13	1·27	1·25	4·86	4·88	4·32	4·335	4·31	4·33	−·005
4. 2	·839	6·555	1·27	1·25	5·285	5·305	4·695	4·71	4·42	4·43	+·09
6. 2	·839	6·26	1·27	1·25	4·99	5·01	4·435	4·45	4·21	4·22	−·12
4. 1	·840	6·62	1·27	1·25	5·35	5·37	4·75	4·77	4·47	4·49	+·155
3. 2	·843	6·475	1·27	1·25	5·205	5·225	4·625	4·64	4·33	4·345	+·015
5. 2	·846	6·315	1·27	1·25	5·045	5·065	4·48	4·50	4·23	4·25	−·075
26. 2	·846	6·17	1·27	1·25	4·90	4·92	4·355	4·37	4·35	4·36	+·035
16. 2	·851	6·24	1·27	1·25	4·97	4·99	4·415	4·435	4·32	4·34	+·02
3. 1	·855	6·42	1·27	1·25	5·15	5·17	4·575	4·59	4·29	4·30	−·01
5. 1	·858	6·36	1·27	1·25	5·09	5·11	4·52	4·54	4·27	4·29	−·02
16. 1	·862	6·18	1·27	1·25	4·91	4·93	4·36	4·38	4·27	4·29	−·01
19. 1	·880	6·22	1·27	1·25	4·95	4·97	4·40	4·415	4·33	4·35	+·07
19. 2	·884	6·18	1·27	1·25	4·91	4·93	4·36	4·38	4·29	4·31	+·04
27. 1	·890	6·08	1·27	1·255	4·81	4·825	4·27	4·285	4·265	4·28	+·015
15. 2	·895	6·13	1·27	1·255	4·86	4·875	4·315	4·33	4·21	4·23	−·03
11. 2	·903	6·32	1·27	1·255	5·05	5·065	4·485	4·50	4·32	4·34	+·095
11. 1	·904	6·315	1·27	1·255	5·045	5·06	4·48	4·495	4·32	4·33	+·09
15. 1	·904	6·07	1·27	1·255	4·80	4·815	4·265	4·28	4·16	4·18	−·06
27. 2	·907	6·03	1·27	1·26	4·76	4·77	4·23	4·24	4·225	4·235	±·00
20. 2	·935	6·12	1·27	1·265	4·85	4·855	4·31	4·315	4·255	4·26	+·06
22. 2	·935	5·96	1·27	1·265	4·69	4·695	4·17	4·17	4·13	4·13	−·07
14. 2	·938	6·275	1·27	1·27	5·005	5·005	4·45	4·45	4·33	4·33	+·135
21. 2	·939	6·02	1·27	1·27	4·75	4·75	4·22	4·22	4·17	4·17	−·02
12. 1	·944	6·225	1·27	1·27	4·955	4·955	4·40	4·40	4·26	4·26	+·075
12. 2	·945	6·16	1·27	1·27	4·89	4·89	4·345	4·345	4·205	4·205	+·025
21. 1	·950	6·055	1·27	1·275	4·785	4·78	4·25	4·245	4·20	4·20	+·025
22. 1	·951	5·98	1·27	1·275	4·71	4·705	4·185	4·18	4·15	4·14	−·035
13. 1	·961	6·16	1·27	1·28	4·89	4·88	4·345	4·335	4·22	4·21	+·05
20. 1	(·966	5·945)	1·27	1·28	(4·675	4·665	4·155	4·145	4·10	4·09)[3]	−·06
13. 2	·967	6·12	1·27	1·28	4·85	4·84	4·31	4·30	4·185	4·175	+·025
14. 1	·974	6·10	1·27	1·29	4·83	4·81	4·29	4·275	4·175	4·16	+·02
										Sum	+·355

[1] Slightly uncertain. [2] Slightly uncertain. [3] Determination rather doubtful.

high quotients first. The uncorrected curves are therefore of little value quantitatively.

Table XVII.

Date April 1918	R.Q. work	Work Cal. b	Rest e_I	Cal. e_{II}	Difference d_I	Difference d_{II}	Cal. per unit work e_I	Cal. per unit work e_{II}	Cal. per unit work e_I corr.	Cal. per unit work e_{II} corr.	Deviation from curve (e_{II} corr.) δ
8.	·732	6·67	1·315	1·33	5·355	5·34	4·76	4·745	4·55	4·53	−·01
9.	·748	6·51	1·29	1·31	5·22	5·20	4·635	4·62	4·44	4·43	−·08
24.	·760	6·455	1·28	1·295	5·175	5·16	4·60	4·585	4·58	4·56	+·06
7.	·763	6·56	1·275	1·29	5·285	5·27	4·695	4·68	4·47	4·46	−·035
23.	·778	6·29	1·27	1·28	5·02	5·01	4·46	4·45	4·43	4·42	−·05
17.	·791	6·51	1·27	1·265	5·24	5·245	4·655	4·66	4·57	4·57	+·115
18.	·800	6·39	1·27	1·26	(5·12	5·13)	4·55	4·56	4·47	4·48[1]	+·04
10.	·804	6·57	1·27	1·26	5·30	5·31	4·71	4·72	4·53	4·54	+·105
5.	·826	6·47	1·27	1·25	5·20	5·22	4·62	4·635	4·365	4·38	−·02
25.	·829	6·20	1·27	1·25	4·93	4·95	4·38	4·40	4·36	4·385	−·01
6.	·830	6·375	1·27	1·25	5·105	5·125	4·535	4·55	4·30	4·32	−·075
4.	·831	6·505	1·27	1·25	5·235	5·255	4·65	4·67	4·37	4·39	±·00
3.	·845	6·47	1·27	1·25	5·20	5·22	4·62	4·635	4·33	4·34	−·035
26.	·848	6·135	1·27	1·25	4·865	4·885	4·32	4·34	4·31	4·33	−·04
16.	·849	6·24	1·27	1·25	4·97	4·99	4·415	4·435	4·32	4·34	−·03
19.	·870	6·24	1·27	1·25	4·97	4·99	4·415	4·435	4·35	4·37	+·03
15.	·882	6·185	1·27	1·25	4·915	4·935	4·365	4·385	4·26	4·28	−·04
27.	·892	6·11	1·27	1·255	4·84	4·855	4·30	4·315	4·30	4·31	±·00
11.	·902	6·36	1·27	1·255	5·09	5·105	4·52	4·535	4·36	4·37	+·075
12.	·917	6·28	1·27	1·26	5·11	5·12	4·54	4·55	4·39	4·40	+·13
20.	·937	6·18	1·27	1·27	4·91	4·91	4·36	4·36	4·30	4·30	+·06
14.	·940	6·185	1·27	1·27	4·915	4·915	4·365	4·365	4·25	4·25	+·01
13.	·944	6·26	1·27	1·27	4·99	4·99	4·435	4·435	4·30	4·30	+·07
22.	·949	6·06	1·27	1·275	4·79	4·785	4·255	4·25	4·22	4·21	−·01
21.	·970	6·00	1·27	1·285	4·73	4·715	4·20	4·19	4·15	4·14	−·05
										Sum	+·21

[1] Slightly uncertain.

The dispersion about the curve of the corrected e_{II} values of the first and second periods is $\sigma = 0{\cdot}06$ Cal. or 1·4 % of the average metabolism $e_{0{\cdot}85} = 4{\cdot}32$ Cal. For the third periods the corresponding dispersion is likewise $\sigma = 0{\cdot}06$ Cal. or 1·35 % of $e_{0{\cdot}85} = 4{\cdot}37$ Cal. As the unavoidable technical errors of the determinations are on an average about 1 %, it is obvious that the variations due to the subject are very slight indeed.

The straight line representing periods 1 and 2 corresponds to an $e_F = 4{\cdot}52$ Cal. and $e_C = 4{\cdot}10$ Cal. The waste of energy from fat is therefore 0·42 Cal. or 9·3 %. The straight line, Fig. 20, representing the third experimental periods corresponds to $e_F = 4{\cdot}57$ Cal. and $e_C = 4{\cdot}15$ Cal. or a waste of energy from fat of 0·42 Cal. = 9·2 %.

THE GENERAL RESULTS OF THE FOUR SERIES OF EXPERIMENTS.

1. *The fatigue at different quotients.*

The subjects J. L., G. L., A. K. and O. H. observed distinct differences in the facility (or difficulty) with which the prescribed amount of work was performed, coincident with changes in diet and noted that on fat diets the fatigue became considerable and sometimes excessive. For several hours after the work on the ergometer these subjects were generally very tired when on a fat diet and much less tired or not tired at all when on carbohydrates. In the notes of O. H. indications are found of a perceptible difference also between work on a mixed diet and on a carbohydrate diet.

The subjects R. E. and A. M. N. failed to observe any appreciable difference between work on different diets. They never became very tired, either during or after the work. As the subjective estimate of the load upon an ergometer is liable to very considerable errors, especially when work done on different days has to be compared, the failure of the two subjects to notice the difference need not mean that such a difference was absent. On the other hand we think it rather probable that work at low quotients has not the same effect upon different individuals. The variations in fatigue may for instance be correlated with individual differences in the liability to acidosis. The clearing up of this point will require a special investigation in which the hydrogen ion concentration of the blood will have to be determined during and after the work.

2. *The waste of energy from fat.*

It is obvious that the objective difference in the economy of work at different quotients cannot be due to any incomplete oxidations at low quotients, since the difference is apparent at all quotients and is on the whole a straight line function of the quotient.

As mentioned above, p. 310, we took as our starting point the hypothesis that fats have to undergo a certain definite transformation involving loss of heat before they can be utilised for the production of muscular energy, and we deduced as necessary consequences of the hypothesis that the curves representing the relation between the expenditure of energy per unit work and the respiratory quotient should be straight lines and should show the same percentage waste of energy from fats in different individuals.

The first of these deductions has been verified by our experiments belonging to the second and fourth series. The best curve for the third series is very nearly a straight line, while in the preliminary series the experiments are too few and too discordant to decide anything quantitatively.

The three straight lines found for O. H. in the second and fourth series of determinations show practically the same waste of energy from fat, viz. 9·8, 9·3 and 9·2 % respectively, but the two straight lines representing the third series on A. M. N. give somewhat higher values, viz. 14·4 and 13·3 %.

We are unable to decide whether the difference between the result of the third series and the other two is real or due to the unavoidable errors with which our determinations of the waste of energy are infested. As the "best" curves for A. M. N. are not absolutely straight there is also the possibility that the metabolism at the lowest quotients has been slightly increased by some special influence. All the determinations (periods 1 and 2) made at quotients above 0·78 can be very accurately represented by a straight line giving $e_F = 4{\cdot}6$ Cal. and $e_C = 4{\cdot}1$ Cal. or $w = 0{\cdot}5$ Cal. $= 10{\cdot}9$ %.

Table XVIII.

Subject and series	Calories per unit work: from fat e_F	Calories per unit work: from carbohydrate e_C	Difference: Cal. w	Difference: % $\frac{100w}{e_F}$	Number of exp. n	Dispersion Cal. σ	Weight $\frac{n}{\sigma}$	$\frac{100wn}{e_F\sigma}$	Average efficiency % $\frac{200}{e_F+e_C}$
J. L.	5·69	4·59	1·10	19·4	10				
G. L.	5·84	5·09	0·75	12·8	15	0·20	75	960	18·3
A. K.	5·04	4·28	0·76	15·1	15	0·21	71	1072	21·6
R. E.	4·72	3·72	1·00	21·2	13	0·155	84	1780	23·7
M. N. Tb. XII	4·70	4·02	0·68	14·5	33	0·074	447	6485	23·0
M. N. Tb. XIII	4·73	4·10	0·63	13·3	18	0·080	225	2993	22·7
O. H. Tb. IX	4·79	4·32	0·47	9·8	33	0·072	459	4500	22·0
O. H. Tb. XVI	4·52	4·10	0·42	9·3	49	0·060	818	7600	23·2
O. H. Tb. XVII	4·57	4·15	0·42	9·2	24	0·060	400	3680	23·0
							Sum 2579	29070	

In Table XVIII we have put together the results obtained in all the different series. There is no definite reason to exclude any of these except the first on J. L. (on account of his illness during the fat diet period) but it is evident that as determinations of the waste of energy from fat their value is very different, and a simple average of the figures in column $\frac{100w}{e_F}$ would be very misleading. We have therefore endeavoured to assign a definite "weight" to each series which should represent its relative reliability, but we are aware that our method of doing so is somewhat arbitrary. We have given the series weight in proportion to the number of determinations in each and further in inverse ratio of the dispersion of the determinations. The approximate weights (nearest whole numbers) are given in column $\frac{n}{\sigma}$, and in column $\frac{100wn}{e_F\sigma}$ we have the products of the weights with the percentage waste of energy found in the series. When the sum of these figures is divided by the sum of the weights we have $\frac{29070}{2579} = 11{\cdot}25$ as the average percentage waste of energy from fat for the whole of our experimental material.

When the preliminary experiments are excluded and the waste of energy observed in the third series is reduced to 11 % by taking into account only those determinations showing quotients above 0·78, the remaining series can be given weights simply in proportion to their number of determinations since their standard deviations will be practically equal and the average works out as $\frac{1456}{148} = 9{\cdot}9$ %.

Table XIX.

Subject and series	Calories per unit work: from fat e_F	Calories per unit work: from carbohydrate e_C	Difference: Cal. w	Difference: % $\frac{100w}{e_F}$	Number of exp. n	$n \times \frac{100w}{e_F}$
M. N. Tb. XII	4·58	4·08	0·50	10·9	27	294
M. N. Tb. XIII	4·68	4·18	0·50	10·7	15	161
O. H. Tb. IX	4·79	4·32	0·47	9·8	33	324
O. H. Tb. XVI	4·52	4·10	0·42	9·3	49	456
O. H. Tb. XVII	4·57	4·15	0·42	9·2	24	221
					148	1456

3. *Possible systematic errors in the determination of the waste of energy from fat. The work of respiration.*

The total amount of work incidental to the production of 1 Cal. external work includes several items which are expended in the organism itself, as the increased circulation and respiration and the sweat secretion. It has been assumed in the calculation that this work is constant and independent of the respiratory quotient. This assumption cannot be proved in any case, and in the case of the respiration it can be shown to be incorrect. It is necessary therefore to examine this point somewhat closely and to see what influence the systematic error introduced may possibly have upon the result.

If at a quotient of 1·00 the amount of work performed by a subject corresponds to an oxygen absorption of 1070 cc. per minute, which represents fairly the conditions in most of our experiments, the oxygen absorption at a quotient of 0·71 will, according to our result, be 1210 cc. The corresponding CO_2 productions will be 1070 and 860 cc. respectively. The elimination of the surplus of CO_2 at the high quotient $1070 - 860 = 210$ cc. will require an increase in ventilation, and on the assumption that the alveolar CO_2 percentage remained the same in both cases the increase could be calculated. Hasselbalch [1912] has shown however that on a carbohydrate diet the CO_2 percentage in the alveoli is somewhat increased, and the increase might possibly be sufficient to make the ventilation constant throughout the whole range of quotients. In any case it makes it impossible to calculate *a priori* the increase in ventilation.

From the work experiments of Frentzel and Reach [1901] (referred to in detail below) in which the ventilation of the lungs was measured at different quotients, Zuntz [1911] has calculated the increase in ventilation incidental to a definite increase in quotient. At an average quotient of 0·778 he finds the ventilation per minute and per Calorie of metabolism to be 4·875 l. while at a quotient of 0·894 it is 5·135 l. or a difference of 0·26 l. At the quotients 0·71 and 1·00 the difference per Calorie would amount to 0·62 l. and in our

experiments with a total metabolism between 6 and 5 Cal. the difference would be 3·5 l.[1]

Zuntz calculates that each litre ventilation corresponds to an oxygen consumption in the respiratory muscles of 5 cc. or 0·025 Cal., but the very careful determinations of the respiration work made by Liljestrand [1917] show that, when the respiration is free to adjust itself, it is much more economically performed, and the probable consumption of energy per litre ventilation is of the order 0·005 to 0·01 Cal. when the ventilation is, as in our experiments, about 25 to 30 l. per minute. The increased cost of eliminating the CO_2 when the quotient rises from 0·71 to 1·00 amounts therefore in our experiments to between 0·02 and 0·03 Cal., or certainly not more than 0·6 % of the total metabolism per Calorie external work. When corrected for the difference in work of breathing the waste of energy from fat is therefore increased by 0·4 to 0·6 %.

The cost of ventilating the lungs is not the same in different individuals, and it is obvious that other activities of the body incidental to the work, *e.g.* the secretion of sweat, need not be the same on carbohydrates as on fat and may vary from one individual to another. The final figure obtained by us for the waste of energy from fat may therefore be influenced by several systematic errors. Seeing that the difference in ventilation, which is very pronounced, causes only an error of about 0·5 % it is very unlikely, however, that the influence of other varying activities can be anything but very slight.

As mentioned on p. 298 we have used the table of caloric values for oxygen at different quotients given by Zuntz in 1913. This table differs somewhat from the table originally computed by Zuntz and Schumburg and shows a higher caloric value for oxygen at low quotients. The difference amounts to 1·7 %, and the waste of energy from fat computed from our experiments will therefore have to be reduced by 1·7 % (from 11·25 to 9·55 %) if the later table is replaced by the earlier. The difference is mainly due to differences in the assumed composition of fat, and it is obvious that so long as the constitution of the fat actually catabolised is unknown the possibility both of a small systematic error and of slight individual differences cannot be avoided.

The Results of Earlier Investigations.

We have compared the result obtained by us with the figures found in earlier investigations to find out how far the actual determinations made—apart from the conclusions drawn—agree or disagree with ours.

A few (5) experiments made on a dog were published in 1894 by Zuntz and Loeb [1894]. They show about the same average metabolism per unit work

[1] The increase in CO_2 production was found above to be 210 cc. or 6 % of the increase in ventilation. The increase in alveolar CO_2 percentage with increasing quotient must therefore have been very slight during the work.

at the different quotients, but the individual results at about the same quotient differ by about 12 % and no valid conclusions can therefore be drawn.

An extensive series of experiments was performed under the supervision of Zuntz by Newton Heineman [1901] on a labourer who worked each day during a period of several months on an "ergostat" which could be turned by hand. The exchange measured varies enormously from day to day—sometimes by 100 %. There is a distinct decrease throughout the period in the metabolism per unit work, from 4·7 Calories per Cal. technical work in February to 4·38 in May according to a calculation by Zuntz [1901]. The main result was that the average metabolism per unit work was lower on a fat diet (4·0 Cal.) than on a diet consisting chiefly of carbohydrates (4·4 Cal.), but Zuntz points out that the difference is probably due to the increasing training as the fat experiments were carried out later than the carbohydrate experiments. In any case the individual determinations are too discordant to allow any definite conclusion.

At about the same time Frentzel and Reach [1901] published another research carried out in the laboratory of Zuntz. These authors acted as subjects themselves, and the work was performed by walking upwards on an inclined treadmill. Each diet was maintained for a week at a time, during which 3–4 periods of work of about 10 minutes duration each were measured each day. In these series the dispersion of individual experiments was much smaller than in Heineman's series, amounting in the most uniform week to $\sigma = 3{\cdot}4$ % and in the least uniform to $\sigma = 12{\cdot}5$ %. The average for each week should therefore be fairly reliable, but unfortunately the two weeks for each subject on the same diet differ about as much from one another as from those on a different diet.

They obtained the following results:

Table XX.

				Average R.Q.	Cal. per kg. per m.	Average
Frentzel.	Fat diet	first week	31. v.–4. vi.	0·766	2·088	2·07
		second week	28. vi.–3. vii.	0·778	2·049	
	Carbohydrate diet	first week	9 vi.–12. vi.	0·896	1·932	1·98
		second week	5. vii.–10. vii.	0·880	2·031	
Reach.	Fat diet	first week	16. vi.–19. vi.	0·805	2·259	2·15
		second week	12. vii.–17. vii.	0·766	2·034	
	Carbohydrate diet	first week	22. vi.–26. vi.	0·899	2·202	2·10
		second week	19. vii.–24. vii.	0·901	2·005	

In both series the metabolism is lower on "carbohydrates" than on fat and when the corresponding differences are calculated for quotients of 0·71 and 1·00 respectively the percentage waste of energy from fat works out as 11 % in the case of Frentzel and 6 % for Reach. It must be conceded however that these results may be due to training, as the carbohydrate experiments

were done after the fat experiments, and in any case the results obtained on the same diet are too discordant to allow any very definite conclusions[1].

Benedict and Cathcart [1913] have made some experiments on the influence of diet in their extensive study of the metabolism during muscular work. They made experiments on a professional bicyclist, who rode on the Atwater-Benedict bicycle ergometer, and determined the respiratory exchange by means of the Benedict "Universal Respiration Apparatus." In the experiments here considered two experimental periods were taken during each ride, and these have been averaged by the authors. Generally the rate of pedalling varied considerably from period to period, and in some cases the respiratory quotients and metabolism per unit work were also very different.

Benedict and Cathcart consider that "the evidence obtained is by no means conclusive" but they have put together such results as are suitable for comparison. In nine experiments the external muscular work was not far from 2 Cal., and in six of these the preceding diet was rich in carbohydrate while in three it was poor in carbohydrate. Comparing the average for the six first-named days with that for the three last they find the same metabolism per unit work (standard metabolism not deducted). When, however, the experiments are arranged according to the quotients actually determined during the work we obtain the following results:

Table XXI.

Date	R.Q.	Cal. per unit work. Gross
Feb. 15	0·95	5·17
Feb. 14	0·93	5·20
Jan. 25	0·92	5·38
Feb. 7	0·91	5·30
Feb. 16	0·88	5·12
Jan. 24	0·87	5·52
Feb. 8	0·86	5·40
Jan. 26	0·83	5·81
Feb. 9	0·82	5·23

It is seen from the plotting given in Fig. 21 that the results are too irregular to admit of any conclusions, but that they are perfectly compatible with our result: that there is a definite waste of energy derived from fat.

In another series of six experiments with a smaller amount of external work there is no apparent difference between the value of carbohydrate and fat, but the discrepancies between the single determinations are even greater than in the series reproduced.

We must mention finally the beautiful experiments made by Anderson and Lusk [1917] on a dog walking horizontally on a treadmill at rates between

[1] It should be remembered that the problem studied by Zuntz and his collaborators was that raised by Chauveau [1898] whether fat must be converted into carbohydrate before being utilised for muscular work. Zuntz calculated that such a conversion must involve an energy loss of at least 24 % and probably 30 % and it must be admitted that the results found by Frentzel and Reach are not compatible with the assumption of such a loss.

3900 and 4800 m. per hour. When the dog had fasted for 18 hours they found an extra energy production over the standard metabolism corresponding to 0·580 kg.meter per kg. of the dog's weight per meter. The respiratory quotient was in one case 0·74, in another 0·78 (average 0·76). After ingestion of 70–100 g. glucose the resting metabolism was increased some 20 % over the standard metabolism during the absorption, but the extra energy required for walking was nevertheless diminished to 0·550 kg.meter per kg. per meter with a quotient of 0·95 (average of three determinations). The difference is a little over 5 % and when the metabolism per unit work is assumed to be a straight line function of the quotient the waste of energy from fats works out as 8 %, without regard to the increase in resting metabolism due to the absorption of the glucose. As the part played by protein in the metabolism is unknown and may have varied it is not possible to make an exact comparison between the result of Lusk and Anderson and ours, but the essential agreement between them is unmistakable.

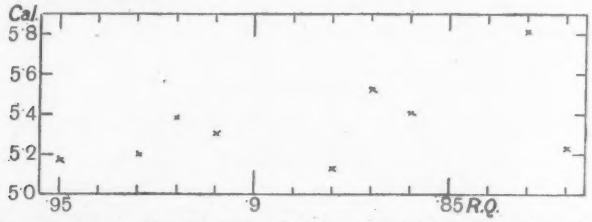

Fig. 21. Experiments from Benedict and Cathcart. Metabolism per unit work, gross.

The foregoing analysis shows that our main numerical result is not at variance with the numerical results of earlier investigations of the same problem and is actually supported by the experiments of Lusk and Anderson and Frentzel and Reach as well as by one series of Benedict and Cathcart's experiments, all of which indicate with more or less probability that the value of fat as a source of muscular energy is lower than that of carbohydrate and that the difference is in the neighbourhood of 10 %.

The Theoretical Significance of the Waste of Energy from Fat.

On the theoretical significance of the difference in value between fat and carbohydrate as a source of energy for the muscular machine, we have very little to say. According to the calculation of Chauveau (quoted by Zuntz) [1911], who assumed that fat was converted into sugar by retaining its hydrogen, splitting of some of the carbon and taking up oxygen to complete the molecule, 30 % of the energy of the fat should become lost by the conversion. It follows therefore from our result—which agrees in so far with the conclusion of Zuntz—that a conversion of this kind does not take place.

Zuntz [1911] has made another calculation, assuming that all the carbon of the fat is retained in the sugar formed. This would correspond to 191·25 g.

sugar formed from 100 g. fat. The fat could yield 950 Cal. and the sugar only 718 Cal.; that is there would be a loss of 24·4 %. This assumption too is incompatible with our determinations.

We are not convinced of the validity of any of these summary methods of calculating the waste of energy incidental to the conversion of fat into sugar or any other substance[1]. We believe that it will be necessary to find out about the stages through which the conversion is actually carried out and the role which may be played by interaction between intermediate products of the fats and other substances which may possibly be derived from carbohydrates.

On the other hand it is clear that our experiments cannot be used as evidence to prove that fat must be converted into sugar before being utilised for muscular work. The chemical processes actually taking place in muscles, doing work upon a fat diet, must be investigated by studies on the lines so brilliantly initiated by Fletcher, Hopkins, Hill and their collaborators, but it is our hope that the result at which we have arrived—that these processes involve a loss of energy of definite magnitude—may serve as a guide in such studies.

APPENDIX I

Statistical Treatment of the Determinations of Standard Metabolism.

We have regularly each day made two consecutive determinations of about five minutes duration of the standard metabolism of our subjects. A statistical treatment of this material can give some information about the accuracy of the method employed and about the influence of various factors upon the results.

In Table XXII we have arranged the repeated determinations of ventilation, respiratory quotient and metabolism in horizontal lines. For each person we have taken the experimental days in the order of the quotients, beginning with the lowest. The material has been arranged primarily to study the possible influence of variations in the pulmonary ventilation upon the quotient and the metabolism. From our earlier experience, especially with the recording respiration apparatus [Krogh, 1913], we know that in periods of a few minutes duration the ventilation during rest is not quite constant but shows oscillations, and this is borne out also by the present series of determinations.

A priori the oscillations in ventilation must be assumed to influence the quotient observed, which should increase with increasing ventilation and *vice versa* by relative washing out and storing up of carbon dioxide, while the oxygen absorption and the metabolism calculated from it should be practically unaffected.

[1] As a reason for suspicion against summary methods of calculating the loss of energy in question we would suggest the making up of 1 molecule of fat (tripalmitin) into 12 molecules of sugar by the addition of 21 molecules of CO_2 and 23 of H_2O which would result in a *gain* of energy amounting to about 18 %.

Table XXII.

Subject and date	Ventilation l. per m. × 100				R.Q. × 100				Calories per m. × 100			
	I			II	I			II	I			II
I	2	3	4	5	6	7	8	9	10	11	12	13
J. L.												
1. ii.	403		+ 8	411	77		+ 4	81	106		0	106
2. ii.	399	− 7		392	81	− 1		80	100	0		100
G. L.												
25. i.	517		+12	529	76		0	76	110		+ 1	111
26. i.	480		+ 9	489	76		+ 4	80	104		− 2	102
26. ii.	532	−59		473	80	− 1		79	104	− 8		96
A. K.												
20. ii.	688		+ 3	691	70		+ 3	73	115		+ 3	118
19. ii.	696	−82		614	72	+ 3		75	122	−18		104
1. iii.	618		+ 2	620	83		+ 5	88	110		0	110
12. ii.	650		+10	660	88		− 1	87	104		+ 1	105
13. ii.	651		+17	668	88		+ 6	94	106		− 3	103
R. E.												
25. iii.	609	−48		561	73	− 2		71	140	− 8		132
26. iii.	548			548	74		+ 2	76	124		+ 4	128
20. iii.	559	−15		544	85	− 2		83	115	− 1		114
6. iv.	640	−35		605	93	− 3		90	134	− 4		130
7. iv.	571		+20	591	93		+ 2	95	125		+ 2	127
	Sums	−246	+81			− 6	+25			−39	+ 6	
A. M. N.												
10. ii.	528			528	75		+ 3	78	112		− 4	108
9. ii.	517	−36		481	78	0		78	112	− 4		108
8. ii.	529		+ 4	533	79		− 2	77	112		+ 4	116
16. ii.	516	−22		494	79	+ 2		81	108	− 4		104
17. ii.	510		+28	538	81		− 2	79	104		0	104
30. i.	615		+23	638	82		+ 2	84	113		− 9	104
7. ii.	500		+ 7	507	83		0	83	113		− 3	110
15. ii.	530	−49		481	83	+ 1		84	106	− 6		100
29. i.	679		+51	730	84		+ 2	86	116		− 5	111
1. ii.	641	−21		620	85	+ 1		86	119	+ 1		120
19. ii.	498		+12	510	86		− 1	85	102		+ 1	103
11. ii.	505		+ 5	510	85		+ 2	87	104		− 2	102
31. i.	620	−23		597	87	0		87	109	− 4		105
6. ii.	580		+ 3	583	88		0	88	114		0	114
2. ii.	600	−40		560	90	− 2		88	122	− 6		116
3. ii.	617	− 6		611	90	− 1		89	118	+ 2		120
12. ii.	546	−33		513	94	− 4		90	108	− 2		106
18. ii.	556	−21		535	97	− 5		92	104	− 2		102
5. ii.	561		+40	601	93		+ 8	101	118		− 3	115
13. ii.	616		+ 3	619	97		+ 2	99	108		+ 2	110
4. ii.	620		+35	655	98		+ 1	99	118		+ 4	122
14. ii.	623	−23		600	106	− 3		103	110	− 2		108
	Sums	−274	+211			−11	+15			−27	−15	

Table XXII *continued.*

Subject and date	Ventilation l. per m. ×100				R.Q. ×100				Calories per m. ×100			
	I			II	I			II	I			II
1	2	3	4	5	6	7	8	9	10	11	12	13
O. H. I												
25. v.	561		+17	578	69		+ 3	72	140		+ 6	146
21.	577	− 7		570	71	− 1		70	144	+ 3		147
24.	622		+13	635	71		+ 2	73	155		− 2	153
22.	594		+31	625	71		+ 4	75	140		0	140
10.	605		+39	644	82		− 1	81	142		0	142
11.	597		+38	635	79		+ 5	84	148		− 2	146
8.	593			593	82		0	82	128		− 2	126
18.	595		+11	606	93		− 4	89	138		+ 4	142
14.	634	−13		621	92	+ 4		96	138	− 8		130
15.	624		+ 4	628	94		0	94	146		− 6	140
17.	616		+27	643	92		+ 7	99	140		− 2	138
	Sums	−20	+180			+ 3	+16			− 5	− 4	
O. H. II												
9. iv.	538	−18		520	64	+ 2		66	147	−10		137
8.	513		+54	567	70		+ 2	72	135		+ 3	138
24	646	−55		591	73	− 1		72	132	− 5		127
18.	552		+29	581	77		+ 2	79	126		+ 1	127
3.	571		+79	650	79		+ 7	86	128		0	128
6.	552	− 2		550	78	+ 2		80	128	− 3		125
4.	595	−63		532	82	− 2		80	134	−16		118
16.	578		+ 2	580	83		− 3	80	126		+ 2	128
5.	563	−24		539	84	− 2		82	132	− 2		130
10.	575	−11		564	83	+ 1		84	124	0		124
15.	636	−17		619	84	+ 2		86	128	+ 2		130
26.	603	−44		559	88	− 6		82	126	− 6		120
23.	532		+32	564	84		+ 3	87	104		+ 6	110
27.	584		+21	605	86		+ 1	87	118		+ 8	126
25.	659	−24		635	86	+ 2		88	127	− 7		120
19.	581	−14		567	90	− 2		88	123	− 1		122
20.	654	−11		643	94	+ 1		95	133	− 2		131
13.	649		+14	663	98		− 4	94	125		+ 8	133
21.	643		+ 9	652	98		0	98	125		+ 7	132
12.	647	−14		633	100	− 3		97	129	+ 2		131
11.	623		+15	638	98		+ 2	100	125		+ 3	128
14.	655		+50	705	101		− 1	100	125		+13	138
	Sums	−297	+305			− 6	+ 9			−48	+51	

In columns 3 and 4 we have arranged the differences between the ventilation in the first and second experimental period (II–I) putting the negative differences in column 3 and the positive in column 4. Though there are individual deviations (notably the first series of experiments on O. H.) we find on the whole that increases in ventilation occur to the same extent as decreases. In 30 cases out of 69 the second period shows a decrease in ventilation amounting on an average to 0·28 l. per minute while in 36 cases

there is an increase amounting on an average to 0·20 l. per minute. As a grand average we have a decrease in ventilation amounting only to 0·016 l. per minute. This means that the introductory period has been of sufficient duration to insure a practically complete quieting down of the ventilation. In the beginning of a period of rest a steady decrease of the ventilation is the general rule.

In the case of O. H. I the almost constant increase in ventilation from the first to the second period is mainly due to a slight inconvenience, caused by the mouthpiece, which became accentuated towards the end of the period and was repeatedly mentioned by the subject.

In columns 6 and 9 we have recorded the quotients found in the first and second period and in columns 7 and 8 the differences (II–I) between them. In column 7 we have put the differences corresponding to a higher ventilation in period I and in column 8 the differences corresponding to a lower ventilation in period I. As most of the differences in column 7 are negative (16 out of 30) and in column 8 positive (25 out of 37), while their sums are respectively negative (− 0·20) and positive (+ 0·65), it follows that an increase in ventilation is correlated, as was to be expected, with an increase in quotient and *vice versa*, but, as the increases show a decided preponderance over the decreases, the final result is an increase in quotient in the second period amounting to 0·0064 ± 0·0036. This increase is nearly double its mean error and is therefore in all probability real. Since the second quotient is the higher we believe that our determinations have generally been made during that period when the probable initial washing out of carbon dioxide is being made good and take it to mean that the resting quotients as determined by us are on an average probably slightly too low.

In columns 10 to 13 we have treated similarly the determinations of metabolism. Here too we find, contrary to our expectations, a distinct correlation with the ventilation. With a decreasing ventilation there is in the greater number of cases (23 out of 30) a fall in metabolism and an increasing ventilation corresponds in 20 cases out of 37 to an increased metabolism. The average decrease in the 30 cases with decreasing ventilation is 0·040 Cal. and the average increase in the 37 cases is 0·010 Cal. On the whole the metabolism in the second period is lower than in the first by 0·0115 ± 0·0062 Cal., a difference which must be assumed to be real.

The correlation between the ventilation and the metabolism might be considered as physiological, since an increase in metabolism must involve an increase in ventilation and *vice versa*. We do not believe, however, that spontaneous oscillations in the metabolism occur during rest within intervals of a few minutes, and think it safer to ascribe the correlation to the technical error in the taking of samples which we have mentioned on p. 303, and which will have just such an effect when the ventilation is not the same in the two consecutive periods.

That the metabolism is on an average lower in the second period than

in the first must probably mean that the preliminary period of rest has not always been long enough for the subject to come down to the true standard metabolism. The difference is very pronounced in the case of A. M. N., where it is on an average $0{\cdot}019 \pm 0{\cdot}008$ Cal., but absent in the case of O. H.: $0{\cdot}002 \pm 0{\cdot}010$ Cal. In future determinations of this kind the preliminary period of rest should be prolonged from $\frac{1}{2}$ to $\frac{3}{4}$ or a whole hour.

The distribution of the variations considered is wholly independent of the respiratory quotient. It is of importance from the point of view of the relation between the quotient and the standard metabolism, treated in Appendix II, that the investigation of the errors affords no ground for assuming that the determinations made at very low or very high quotients should be less reliable or infested with different systematic errors from those at intermediate quotients[1].

When the two determinations made each day are treated as simple repetitions the mean error of their averages which can be called their technical error is determined by $\tau = \frac{1}{2}\sqrt{\frac{\Sigma d^2}{n-1}}$, where d is the difference between each pair of determinations.

We find

Table XXIII.

	R.Q. τ	Cal. per min. τ
I. L.-R. E.	0·016	0·031
A. M. N.	0·014	0·020
O. H. I	0·019	0·021
O. H. II	0·015	0·033
All experiments	0·015	0·027

The technical error of a double determination of the quotient is therefore $\tau = 0{\cdot}015$ and of the metabolism $\tau = 0{\cdot}027$ Cal. or about 2 % of the standard metabolism of our subjects.

APPENDIX II

The Correlation between the Respiratory Quotient and the Standard Metabolism.

In discussing the different series of experiments the standard metabolism has been treated for each subject separately and it has been shown that at very low quotients there is a slight increase in metabolism, while in some experiments a tendency towards an increase at high quotients is also apparent.

In order to obtain more definite information upon this point we have compared all our available data concerning the standard metabolism at

[1] The ventilation shows as might be expected a distinct increase with increasing quotient corresponding to the increasing elimination of carbon dioxide. As we have not counted the respirations and cannot therefore calculate the average depth of respiration or the alveolar ventilation a detailed study of the relation between the ventilation of the lungs and the elimination of carbon dioxide is scarcely worth attempting.

varying quotients. As the metabolism varies with the subject a direct comparison cannot be instituted, but we have calculated the average standard metabolism for each person and determined the percentage deviation from this average for each experimental day. In the case of A. M. N., whose standard metabolism showed a steady decline throughout the three weeks' experiments, we have taken the curve, Fig. 12, as our unit and determined the percentage deviation of each day's result from this curve.

All the figures thus obtained have been plotted in Fig. 22 with the respiratory quotient as abscissa and using different symbols for the different subjects as indicated. In drawing the curve representing the determinations the two lowest values at the quotients 0·82 and 0·845 have been disregarded as probably erroneous.

The curve shows unmistakably and without any apparent difference between the different subjects that the standard metabolism in subjects with a very low protein metabolism, when calculated on the basis of the caloric values for oxygen given by Zuntz, is not independent of the quotient. It has a minimum value between the quotients 0·8 and 0·94, increases by about 5 % when the quotient falls from 0·8 to 0·71 and increases also by about 3 % when the quotient rises from 0·94 to unity. Even between 0·8 and 0·94 it does not appear to be quite constant, but the variation found is only 1 % and is within the limits of error. The dispersion of the single determinations about the curve is $\sigma = 3{\cdot}0$ %. As the mean technical error of a double determination is according to Appendix I $\tau = 2$ % the individual day to day variations of each subject's standard metabolism (ϕ), which is found from the equation $\tau^2 + \phi^2 = \sigma^2$, works out as $\phi = 2{\cdot}2$ %.

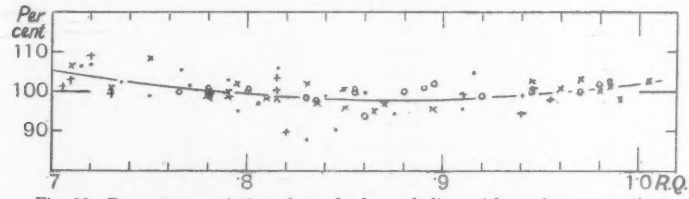

Fig. 22. Percentage variation of standard metabolism with respiratory quotient. Subjects: • J. L., G. L., A. K., R. E., o A. M. N., + O. H. 1917, × O. H. 1918.

A definite explanation of the influence of the materials catabolised upon the standard metabolism, cannot at present be given. It is conceivable, though in our opinion very improbable, that it may be unreal and due to imperfections in the indirect calorimetry employed by us. To settle this point it would be very desirable to have a sufficient number of direct calorimetric determinations of the standard metabolism of human subjects over the whole practicable range of quotients.

When the variation of the metabolism with the quotient must be accepted as real it might be thought to be due at low quotients to incomplete oxidations with excretion of keto-substances. We have repeatedly tested the urine of

Table XXIV.

Date and subject	R.Q.	Cal.	Deviation from average: Cal.	Deviation from average: %	Deviation from curve
J. L.					[3]
1917					
1. ii.	·79	1·055	+0·03	+ 3	+3
2. ii.	·805	·995	−0·03	− 3	−2
	Average	1·025			
G. L.					
25. i.	·765	1·105	+0·06	+ 5·5	+4·5
26. i.	·78	1·035	−0·01	− 1	−1
26. ii.	·795	·995	−0·05	− 5	−4·5
	Average	1·045			
A. K.					
20. ii.	·715	1·17	+0·07	+ 6·5	+2·5
19. ii.	·735	1·13	+0·03	+ 2·5	0
5. ii.	·77	1·115	+0·015	+ 1·5	+1
1. iii.	·86	1·10	±0	0	+2
12. ii.	·875	1·04	−0·06	− 5·5	−3
13. ii.	·91	1·05	−0·05	− 4·5	−2·5
	Average	1·10			
R. E.					
25. iii.	·72	1·36	+0·09	+ 7	+3
26. iii.	·75	1·26	−0·01	− 1	−2·5
20. iii.	·845	1·145	−0·125	−10	−8
6. iv.	·915	1·325	+0·055	+ 4·5	+6·5
7. iv.	·94	1·26	−0·01	− 1	0
	Average	1·27			
A. M. N.					
1918					
10. ii.	·765	1·105	±0[1]	0	−1
8. ii.	·78	1·14	+0·015	+ 1·5	+1·5
9. ii.	·78	1·105	−0·01	− 1	−1
16. ii.	·80	1·055	+0·005	+ 0·5	+1
17. ii.	·80	1·04	±0	0	+0·5
30. i.	·83	(1·085)	(−0·125)	(−10·5)[2]	
7. ii.	·83	1·115	−0·02	− 2	0
15. ii.	·835	1·035	−0·02	− 2	0
29. i.	·85	(1·135)	(−0·085)	(− 7)[2]	
1. ii.	·855	1·195	+0·005	+ 0·5	+2·5
19. ii.	·855	1·02	±0	0	+2
11. ii.	·86	1·03	−0·065	− 6	−4
31. i.	·87	(1·07)	(−0·13)	(−11)[2]	
6. ii.	·88	1·145	±0	0	+2·5
2. ii.	·89	1·19	+0·01	+ 1	+3
3. ii.	·895	1·19	+0·02	+ 2	+4
12. ii.	·92	1·07	−0·015	− 1·5	+0·5
18. ii.	·945	1·03	±0	0	0
5. ii.	·97	1·16	+0·005	+ 0·5	0
13. ii.	·98	1·095	+0·02	+ 2	+1
4. ii.	·985	1·20	+0·035	+ 3	+2
14. ii.	1·045	1·085	+0·02	+ 2	

[1] Deviations from curve, Fig. 12.

[2] Very doubtful. See p. 325.

Table XXIV *continued.*

Date and subject	R.Q.	Cal.	Deviation from average		Deviation from curve
			Cal.	%	
O. H. I					δ
1917					
25. v.	·705	1·43	+0·02	+ 1·5	±3
21.	·71	1·45	+0·04	+ 3	−1
24.	·72	1·54	+0·13	+ 9	+5·5
22.	·73	1·40	−0·01	− 0·5	−3·5
10.	·815	1·42	+0·01	+ 0·5	+1·5
11.	·815	1·465	+0·055	+ 3·5	+4·5
8.	·82	1·27	−0·14	−10	−8·5
18.	·91	1·40	−0·01	− 0·5	+1·5
14.	·94	1·335	−0·075	− 5·5	−4·5
15.	·945	1·425	+0·015	+ 1	+2
17.	·955	1·385	−0·025	− 2	−1·5
	Average	1·41			
O. H. II					
1918					
9. iv.	·65	(1·42)	(+0·14)	(+11)[1]	
8.	·71	1·365	+0·085	+ 6·5	+2·5
24.	·73	1·295	+0·015	+ 1	−2
7.	·75	1·39	+0·11	+ 8·5	+7
18.	·78	1·27	−0·01	− 1	−1
3.	·79	1·28	±0	0	0
6.	·79	1·265	−0·015	− 1	−1
17.	·795	1·31	+0·03	+ 2·5	+3
4.	·81	1·26	−0·02	− 1·5	−0·5
16.	·815	1·265	−0·015	− 1	0
5.	·83	1·31	+0·03	+ 2·5	+4
10.	·835	1·245	−0·035	− 2·5	−1
15.	·85	1·285	+0·005	+ 0·5	+2·5
26.	·85	1·23	−0·05	− 4	−2
23.	·855	(1·07)	(−0·21)	(−16·5)[2]	
27.	·865	1·22	−0·06	− 4·5	−2·5
25.	·87	1·24	−0·04	− 3	−0·5
19.	·895	1·225	−0·055	− 4·5	−2·5
20.	·945	1·32	+0·04	+ 3	+3·5
13.	·96	1·29	+0·01	+ 1	+1
22.	·97	1·32	+0·04	+ 3	+2·5
21.	·98	1·28	±0	0	−1
12.	·985	1·30	+0·02	+ 1·5	+0·5
11.	·99	1·26	−0·02	− 1·5	−2·5
14.	1·005	1·32	+0·04	+ 3	+0·5
	Average	1·28			

[1] Somewhat doubtful.

[2] Considered as erroneous. See p. 333.

our subjects for aceto-acetic acid and found no evidence of acidosis, and the rise in metabolism begins at quotients which are too high to admit of acidosis.

In our opinion the higher metabolism both at low and at high quotients is most probably due to transformation processes of carbohydrate into fat at high quotients and of fat (and protein) into carbohydrate at low quotients. Such transformations will cause at high quotients an increase in the total respiratory quotient over that of the true catabolic processes and at low quotients a decrease, provided of course that the transformation products are stored, at least provisionally.

It is significant therefore that we have occasionally observed quotients above unity, when the subjects were on a carbohydrate diet, and below 0·71, when the diet consisted chiefly of fat. We have no reason to doubt the reality of these quotients, which must mean that synthetic transformations take place, and it is a natural inference that the same transformations take place, though with diminished intensity, when the quotient is somewhat below 1·00 or above 0·71.

Additional evidence corroborating the view that a transformation of fat into carbohydrate takes place regularly, when the respiratory quotient is below 0·8, and the reverse transformation, when the quotient is above 0·9, will be given in Appendix III.

APPENDIX III

The Change in Respiratory Quotient taking place on the Transition from Rest to Muscular Work.

In Table XXV we have compared the average respiratory quotient found each day during rest with the quotient observed on the same day during the first experimental period of work. The experiments have been arranged as usual in the order of increasing quotients during rest.

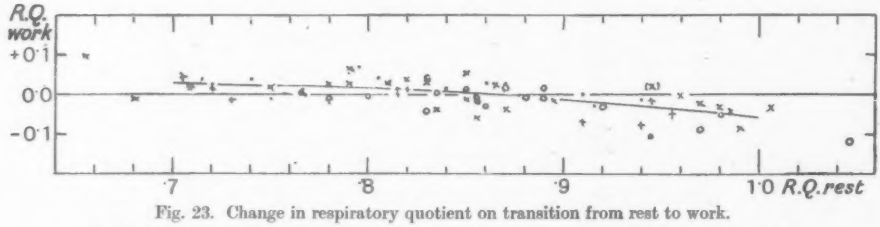

Fig. 23. Change in respiratory quotient on transition from rest to work.
Subjects: • J. L., G. L., A. K., R. E., o A. M. N., + O. H. 1917, × O. H. 1918.

The results have been plotted in Fig. 23 with the quotient during rest as abscissa and the differences in quotient between rest and work as ordinates. When the quotient during work is increased over that during rest the difference has been denoted as positive. Though the individual results are somewhat

irregular it is unmistakable that at low quotients there is on the whole an increase in the quotient on the transition from rest to work, but at high (above 0·9) there is a very decided decrease, amounting, when the quotient during rest approaches unity, to about 0·05.

The dispersion of the individual results about the curve drawn is $\sigma = \pm 0{\cdot}028$ which is partly explained by the technical errors of the determinations involved. The technical error on the difference between resting and working quotient here considered is about $\tau = \pm 0{\cdot}016$. Those variations from the curve, which are due to the subjects, are therefore $\phi = \pm 0{\cdot}023$.

There are two known sources of systematic error in the comparison here given between the respiratory quotients during rest and work. The first is that we have compared the quotient during rest, not with the quotient at the beginning of the work, but with the quotient found during a period extending from 30 to 50 (or 60) minutes after the beginning of work. According to the results given in Appendix IV we must assume that the quotient at the beginning of the work was generally a little higher and by making a somewhat uncertain extrapolation we find it to be about 0·01 higher. A correction of this magnitude would shift the curve slightly to the right so that we should have the zero point at 0·91 instead of at 0·87.

Another systematic error is that the quotients determined during rest are (as shown in Appendix I) probably on an average slightly too low, which may possibly compensate the error on the quotients during work.

Whether the curve shall finally be shifted slightly to the right or slightly to the left the essential result remains unchanged: that on the transition from rest to constant work of moderate intensity a rise in quotient takes place only when the quotient was low beforehand, but a fall when it was high, while at intermediate quotients the average change is very slight.

Our result as here summarised agrees with the findings of Zuntz and his school, but is strikingly at variance with the results reported by Benedict and Cathcart [1913], who found that the quotients during work were generally higher than in a preceding period of rest and were increased more or less in proportion to the severity of the work performed. The quotients determined by Benedict and Cathcart during work have been adversely criticised by Lindhard [1915] on the ground that some of them are undoubtedly influenced by forced respiration and that there is good reason to think that they are generally so influenced on account of the inconvenience of breathing through a mouthpiece and into a respiration apparatus. We would point out further, that since it can be deduced from the experiments of Carpenter [1915], referred to above (p. 293), that the "unit" apparatus employed gives quotients during rest which are on an average 0·03 too high, it is quite possible that during work there is an even greater systematic error.

To explain the increase in standard metabolism both at low and at high quotients we have in Appendix II offered the hypothesis that synthetic processes take place, resulting respectively in the formation of carbohydrate or

Table XXV.

Date	R.Q. rest	R.Q. work	Diff.	Deviation from curve 100 δ
J. L.		•		
1. ii.	·79	·846	+·055	+ 4
2. ii.	·805	·846	+·04	+2·5
G. L.		•		
25. i.	·765	·766	0	− 2
26. i.	·78	·762	−·02	−3·5
26. ii.	·795	·863	+·07	+5·5
A. K.		•		
20. ii.	·715	·757	+·04	+ 1
19. ii.	·74	·781	+·04	+1·5
1. iii.	·86	·891	+·03	+2·5
12. ii.	·87	·896	+·025	+2·5
13. ii.	·91	·909	0	+ 1
R. E.		•		
25. iii.	·72	·747	+·025	0
26. iii.	·75	·742	−·01	− 3
20. iii.	·84	·854	+·015	+0·5
6. iv.	·915	·891	−·025	− 1
7. iv.	·94	·932	−·01	+ 1
A. M. N.		o		
10. ii.	·765	·770	+·005	−1·5
9. ii.	·78	(·768)	−·01[1]	− 3
8. ii.	·78	·770	−·01	−2·5
16. ii.	·80	·793	−·005	− 2
30. i.	·83	·873	+·045	+3·5
7. ii.	·83	·787	−·045	−5·5
15. ii.	·835	·841	+·005	−0·5
29. i.	·85	·866	+·015	+ 1
1. ii.	·855	·850	−·005	− 1
19. ii.	·855	·844	−·01	−1·5
11. ii.	·86	·829	−·03	−3·5
31. i.	·87	·890	+·02	+ 2
6. ii.	·88	·876	−·005	−0·5
2. ii.	·89	·886	−·005	0
3. ii.	·89	·910	+·02	+2·5
12. ii.	·92	·889	−·03	−1·5
18. ii.	·945	·843	−·10[2]	− 7
5. ii.	·97	·882	−·09	−5·5
13. ii.	·98	·930	−·05	− 1
14. ii.	1·045	·926	−·12	

Date	R.Q. rest	R.Q. work	Diff.	Deviation from curve 100 δ
O. H. I		+		
25. v.	·705	·744	+·04	+ 1
21.	·71	·731	+·02	− 1
24.	·72	·735	+·015	− 1
22.	·73	·717	−·015	− 4
10.	·815	·814	0	− 1
11.	·815	·830	+·015	+0·5
8.	·82	·836	+·015	+0·5
18.	·91	·839	−·07	− 6
14.	·94	·864	−·075	− 5
15.	·945	·929	−·015	+ 1
17.	·955	·904	−·05	− 2
O. H. II		×		
9. iv.	·655	·751	+·095	
8.	·71	·728	+·02	− 1
7.	·75	·768	+·02	0
18.	·78	·808	+·03	+1·5
3.	·79	·855	+·065	+ 5
6.	·79	·819	+·03	+1·5
4.	·81	·840	+·03	+ 2
16.	·82	·862	+·04	+ 3
5.	·83	·858	+·03	+ 2
10.	·835	·797	−·04	− 5
15.	·85	·904	+·055	+ 5
26.	·85	·838	−·01	−1·5
23.	·855	·796	−·06	−6·5
27.	·865	·890	+·025	+2·5
25.	·87	·832	−·04	− 4
19.	·895	·880	−·015	− 1
20.	·945	(·966)	(+·02)[3]	
13.	·96	·961	0	+ 3
22.	·97	·951	−·02	+1·5
21.	·98	·950	−·03	+ 1
12.	·985	·944	−·04	0
11.	·99	·904	−·085	− 4
14.	1·005	·974	−·03	+ 2
			Sum	− 25

[1] Slightly uncertain. [2] Slightly uncertain.
[3] Doubtful.

fat with consequent lowering or raising of the respiratory quotient. The quotient of the catabolic processes alone would, according to this view, be higher when the total quotient is low and lower when it is high. When work is being performed the anabolic processes may continue unaltered or may possibly become reduced, because the necessary blood supply is deflected to the working muscles, but there is no reason to think that they should be intensified in proportion to the work. If we assume with Zuntz that the

proportion between the catabolism of carbohydrate and fat remains on the whole unchanged on the transition from rest to work, while the anabolic processes going on at the time are continued without any increase proportional to the increased catabolism, their influence upon the total quotient becomes diminished, and we shall consequently obtain a rise in total quotient when it was low before the work and a fall when it was high—or just what we have found.

The theoretical conception to which our experiments appear to lead up is therefore the following, which is meant not as a proved theory but as a definite formulation of a working hypothesis.

The proportion of carbohydrate to fat catabolised is a function of the relative available quantities of the two substances, and substantially the same during rest and during muscular work.

Fat is formed from carbohydrate and stored up when the available supply of carbohydrate is in excess of the supply of fat. The formation becomes distinct at quotients above 0·9. When the total quotient during rest has reached unity the catabolic quotient is only about 0·95. The conversion of carbohydrate into fat takes place with a loss of energy and a consequent increase of a few per cent. in the total standard metabolism at high quotients.

Sugar (or substances allied to carbohydrates) is formed from fat (and protein) and stored up when the available supply of fat is in excess of the supply of carbohydrate. The formation becomes distinct at quotients below 0·8. When the total quotient during rest has reached 0·71 the catabolic quotient is about 0·74. The conversion of fat takes place with a loss of energy and a consequent increase in the standard metabolism at decreasing quotients.

It is a consequence of this hypothesis that neither fats alone nor carbohydrates alone are suitable for the supply of the energy requirements of the body, but that the catabolic disintegration of either of these substances requires the presence and the simultaneous catabolisation of the other.

While it has long been concluded from the acidosis and excretion of keto-substances in severe diabetes and during starvation that carbohydrates are necessary for the complete catabolisation of fat and our results only lend support to this view, it is, so far as we are aware, a new hypothesis that fats are likewise necessary for the catabolisation of carbohydrate.

APPENDIX IV

The Variation of the Respiratory Quotient and the Metabolism during one hour of Constant Work.

In Table XXVI we have arranged the three work determinations of each day in horizontal lines and figured out the differences in respiratory quotient and total metabolism between the three periods. For each subject we have taken the days in the order of increasing quotients. For the sake of convenience we have multiplied the quotients by 1000 and the Calories per minute

Table XXVI.

Date	R.Q. during work × 1000					Metabolism during work Cal. × 100				
	I	Diff. II–I	II	Diff. III–II	III	I	Diff. II–I	II	Diff. III–II	III
J. L.										
16. i.	767	+ 4	771	+20	791	608	– 3	605	+11	616
15. i.	772	–16	756	+ 4	760	592	+15	607	+39	646
1. ii.	846	+27	873	– 5	868	568	0	568	+ 6	574
2. ii.	846	– 3	843	– 7	836	584	+ 3	587	– 5	582
G. L.										
26. i.	762	– 5	757	+ 5	762	479	+ 2	481	+ 7	488
25. i.	766	+11	777	–26	751	463	+ 8	471	+19	490
26. ii.	863	– 6	857	–24	833	459	+19	478	–10	468
18. i.	906	–21	885	–18	867	428	+17	445	0	445
19. i.	927	–14	913	–13	900	472	– 6	466	–18	448
A. K.										
20. ii.	757	+ 5	762	– 5	757	590	+ 9	599	+ 8	607
19. ii.	781	– 7	774	–19	755	531	+19	550	– 1	549
1. iii.	891	–21	870	–24	846	530	+24	554	+ 9	563
12. ii.	896	–29	867	– 6	861	522	+ 6	528	+ 8	536
13. ii.	909	–16	893	–10	883	538	+ 3	541	+19	560
R. E.										
26. iii.	742	–12	730	+ 8	738	576	+11	587	+15	602
25. iii.	747	– 5	742	+ 9	751					
20. iii.	854	–18	836	–19	817	538	0	538	+ 6	544
6. iv.	891	+ 4	895	–16	879	523	– 1	522	+ 1	523
7. iv.	932	–14	918	–21	897	532	+ 9	541	+25	566
A. M. N.										
9. ii.	(768)	– 4	764	– 4	760	(512)	+25	537	– 1	536
8. ii.	770	– 9	761	+ 3	764	536	+ 2	538	0	538
10. ii.	770	– 7	763	– 2	761	527	+ 2	529	+ 9	538
7. ii.	787	– 5	782	+23	805	514	+11	525	– 6	519
16. ii.	793	–14	779	+23	802	516	+11	527	–14	513
11. ii.	829	+ 1	830	– 5	825	511	+ 1	512	+10	522
15. ii.	841	– 3	838	+ 4	842	499	+ 7	506	+ 5	511
18. ii.	(843)	–14	829	+ 4	833	(491)	+ 5	496	+10	506
19. ii.	844	+ 2	846	– 6	840	495	+ 6	501	+ 1	502
1. ii.	850	– 4	846	0	846	529	+ 7	536	+11	547
29. i.	866	– 6	860	–12	848	612	–14	598	+ 9	607
30. i.	873	–11	862	– 7	855	533	– 3	530	+13	543
6. ii.	876	0	876	– 8	868	511	– 2	509	+11	520
5. ii.	882	+25	907	–31	876	519	– 3	516	+10	526
2. ii.	886	–16	870	+ 2	872	520	+ 7	527	+ 6	533
12. ii.	889	– 5	884	– 8	876	496	– 1	495	+12	507
31. i.	890	– 2	888	– 5	883	520	+ 4	524	+ 8	532
3. ii.	910	–14	896	– 4	892	568	– 5	563	+ 5	568
14. ii.	926	– 2	924	+16	940	514	– 4	510	+ 5	515
13. ii.	930	– 5	925	+ 3	928	493	+ 3	496	+13	509

Table XXVI *continued.*

Date	R.Q. during work × 1000					Metabolism during work Cal. × 100				
	I	Diff. II–I	II	Diff. III–II	III	I	Diff. II–I	II	Diff. III–II	III
O. H. I										
22. v.	717	− 8	709	+10	719	671	− 1	670	+ 8	678
21.	731	−16	715	− 1	714	682	+ 3	685	+ 3	688
24.	735	− 1	734	+31	765	656	+ 7	663	−21	642
25.	744	−13	731	+15	746	668	0	668	0	668
10.	814	−11	803	+ 7	810	682	− 1	681	+ 1	682
11.	830	+ 5	835	−21	814	669	− 8	661	+11	672
8.	836	− 7	829	−11	818	686	− 3	683	+ 4	687
18.	839	−22	817	−18	799	656	+12	668	+ 2	670
14.	864	+ 6	870	+ 6	876	668	− 8	660	− 7	653
17..	904	− 8	896	−15	881	634	+11	645	+ 5	650
15.	929	− 7	922	− 3	919	650	+ 4	654	− 3	651
O. H. II										
8. iv.	728	+12	740	− 8	732	664	− 8	656	+11	667
9.	751	−14	737	+11	748	644	+14	658	− 7	651
7.	768	− 2	766	− 3	763	649	+ 3	652	+ 4	656
23.	796	− 8	788	−10	778	611	+10	621	+ 8	629
10.	797	+ 7	804	0	804	650	− 6	644	+13	657
18.	808	0	808	− 8	800	627	+ 1	628	+11	639
17.	816	− 5	811	−20	791	627	+ 3	630	+21	651
6.	819	+20	839	− 9	830	636	−10	626	+11	637
25.	832	−10	822	+ 7	829	614	+ 3	617	+ 3	620
26.	838	+ 8	846	+ 2	848	613	+ 4	617	− 4	613
4.	840	− 1	839	− 8	831	662	− 7	655	− 5	650
3.	855	−12	843	+ 2	845	642	+ 5	647	0	647
5.	858	−12	846	−20	826	636	− 5	631	+16	647
16.	862	−11	851	− 2	849	618	+ 6	624	0	624
19.	880	+ 4	884	−14	870	622	− 4	618	+ 6	624
27.	890	+17	907	−15	892	608	− 5	603	+ 8	611
11.	904	− 1	903	− 1	902	631	+ 1	632	+ 4	636
15.	904	− 9	895	−13	882	607	+ 6	613	+ 5	618
12.	944	+ 1	945	−28	917	622	− 6	616	+12	628
21.	950	−11	939	+31	970	605	− 3	602	− 2	600
22.	951	−16	935	+14	949	598	− 2	596	+10	606
13.	961	+ 6	967	−23	944	616	− 4	612	+14	626
20.	(966)	−31	935	+ 2	937	(594)	+18	612	+ 6	618
14.	974	−36	938	+ 2	940	610	+17	627	− 9	618

by 100 so as to have the differences in whole numbers. In the experiments on J. L., G. L., A. K. and R. E. the periods were of about 30 minutes duration; in the first series of experiments on O. H. they were of about 24 minutes and in the remainder of the experiments of 21 minutes duration.

The table shows that the quotient varies irregularly but on the whole very slightly. In most cases there is a slight decrease in quotient from the first to the second and again from the second to the third period but it does not amount to more than about 0·01, and in some cases there is an increase of similar magnitude. The largest difference observed between two periods

is 0·035. We have averaged the differences found in all the experiments (74) and those found in the most reliable series with periods of about equal length (series 2–4) with the following results:

Number	R.Q.II–R.Q.I	R.Q.III–R.Q.II	Cal. II–I	Cal. III–II
74	−0·0056	−0·0040	+0·033	+0·054
55	−0·0051±0·0014	−0·0025±0·0018	+0·019±0·010	+0·047±0·010

There is a slight fall in the quotient from the first to the second period and a still smaller from the second to the third. Though the work remains constant the metabolism increases slightly from the first to the second period and somewhat more from the second to the third. As the fall in quotient must cause an increase in the metabolism a correction can be applied to give the change in metabolism from period to period, on the assumption that the quotient remained constant. According to the average result of our determinations a decrease in the quotient of 0·01 will correspond approximately to an increase in the metabolism of 0·02 Cal. In the 55 best experiments the corrected increase in metabolism from the first to the second period will be therefore $0{\cdot}019 - 0{\cdot}51 \times 0{\cdot}02 = 0{\cdot}009 \pm 0{\cdot}010$ and from the second to the third $0{\cdot}047 - 0{\cdot}25 \times 0{\cdot}02 = 0{\cdot}042 \pm 0{\cdot}010$.

The corrected increase in metabolism from period to period must be taken to show the influence of fatigue, and the calculations show that such an influence is practically absent in the second period but distinct, though small, in the third.

In order to see whether the diet has any influence upon the variation of the quotient during work and upon the onset of fatigue we have averaged the differences in the experiments with initial quotients between 0·7 and 0·85 and compared them with the averages derived from the remaining experiments with quotients between 0·85 and 1·00. The results are as follows:

Number	R.Q.	R.Q.II–R.Q.I	R.Q.III–R.Q.II	Cal. II–I	Cal. III–II
28	0·7 – 0·85	−0·0043±0·0017	−0·0001±0·0024	+0·031±0·014	+0·032±0·016
27	0·85 – 1·00	−0·0059±0·0023	−0·0049±0·0025	+0·007±0·014	+0·062±0·012

Caloric difference corrected for decrease in quotient

R.Q.	Cal. II–I	Cal. III–II
0·7 – 0·85	+0·022±0·018	+0·032±0·021
0·85 – 1·00	−0·005±0·017	+0·052±0·017

The fall in quotient during a one hour period of constant work is slightly larger at high quotients than at low, and the influence of fatigue upon the economy of the work appears to be very nearly the same at all quotients. All the changes observed are scarcely outside the limits of error.

In our experiments work has been performed at the same rate for 30 minutes before the beginning of the first period. An extrapolation shows that at the beginning of work the respiratory quotient has probably on an average been about 0·01 higher than in the first experimental period.

The results here given show that the organism maintains during work a remarkably constant proportion between the amounts of carbohydrate and

fat catabolised. This proportion is evidently a function of the available supplies of the two sources of energy, and the change in quotient shows that the balance between carbohydrate and fat is very slowly altered as the work progresses in favour of the latter.

Summary.

1. Methods are described for determining in 20 minute periods the respiratory exchange of human subjects doing constant work on an ergometer placed in a Jaquet respiration chamber.

2. The technical error on determinations of total metabolism made by these methods is below 1 % and the respiratory quotient can be determined with a maximum error of 0·005.

3. Four series of experiments (about 220 determinations) on six different subjects, living during the experiments on a diet poor in protein, have been made to study the relative value of fat and carbohydrate as a source of muscular energy. All the series agree in showing that work is more economically performed on carbohydrate than on fat. When the work was sufficiently severe the subjects performed it with greater difficulty on fat than on carbohydrate and became much more tired.

4. The net expenditure of energy (standard metabolism deducted) necessary to perform one Calorie technical work on the ergometer has varied between about 5·5 and 4 Cal. At a constant quotient it varies with the subject and for the same subject it decreases with increasing training. During one hour of work it generally rises slightly from fatigue.

5. For the single subject and on a constant level of training the relation between the respiratory quotient and the net expenditure of energy per unit work can be expressed graphically as a straight line. Since the proportion of fat to carbohydrate catabolised is also a straight line function of the quotient the difference in value for muscular work between fat and carbohydrate can be expressed by a single figure: the waste of energy from fat. In the three best series of experiments the net expenditure of energy per Cal. technical work varies from about 4·6 Cal. when fat alone is catabolised (R.Q. = 0·71) to about 4·1 Cal. when carbohydrate alone is catabolised (R.Q. = 1·00). The waste of energy from fat is 0·5 Cal. or 11 % of the heat of combustion of the fat.

6. The standard metabolism (during rest, in the postabsorptive state) of a human subject is not independent of the preceding diet. When the diets are poor in protein it is lowest at intermediate quotients and increases about 5 % when the quotient falls to about 0·71 and about 3 % when the quotient rises to about unity.

7. On the transition from rest to moderate muscular work the respiratory quotient is generally altered. On an average it is increased when the quotient was low and diminished when it was high before the work. The fall at high quotients is greater (0·05) than the increase at low (0·03). At quotients between 0·8 and 0·9 the average change on the transition to work is very slight.

8. It is suggested as a working hypothesis:

that both during rest and during work the proportion of fat to carbohydrate catabolised is a function of the available supplies of these substances;

that carbohydrate is formed from fat and provisionally stored when the quotient is below 0·8 while a corresponding transformation of carbohydrate to fat takes place when the quotient is above 0·9;

that these anabolic processes make the total respiratory quotient lower than the catabolic when this is low and higher when it is high and that they give rise to an extra expenditure of energy during rest;

and finally that during work the anabolic processes (combined with storage) are not increased in proportion to the catabolic, whereby the total quotient is lowered when it was high and raised when it was low beforehand.

9. During one hour of constant work of moderate intensity the respiratory quotient decreases on an average only 0·008; slightly more at high quotients and less at low. The metabolism per Cal. technical work increases a little (0·065 Cal.). Part of the increase is due to the fall in quotient (0·01 Cal.) and the rest to fatigue. The effect of fatigue upon the economy of the work appears to show itself earlier when the quotient is low than when it is high.

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REFERENCES.

Anderson and Lusk (1917). *Biol. Chem.*, **32**, 421.
Benedict and Cathcart (1913). *Carnegie Publication*, No. 181.
—— and Roth (1918). *Proc. National Acad. Sc.*, **4**, 149.
Carpenter, T. M. (1915). *Carnegie Publication*, No. 216.
Chauveau (1898). *Compt. Rend.*, **125**, 1070; **126**, 795.
Fletcher and Hopkins (1917). *Proc. Roy. Soc.* B., **89**, 444.
Frentzel and Reach (1901). *Pflüger's Arch.*, **83**, 477.
Grafe (1910). *Zeitsch. physiol. Chem.*, **65**, 1.
Hasselbalch (1912). *Biochem. Zeitsch.*, **46**, 403.
Heineman (1901). *Pflüger's Arch.*, **83**, 441.
Krogh (1913). *Skand. Arch. Physiol.*, **30**, 375.
—— (1915, 1). *Abderhalden's Handb. biochem. Arbeitsmethoden*, **8**, 537.
—— (1915, 2). The Respiratory Exchange of Animals and Man, *Monographs of Biochemistry*, Longmans, Green and Co., London
—— (1915, 3). *J. Physiol. Proc.*, **49**, xxxi.
—— (1919). *K. Danske Vidsk. Selsk. Math.-fys. Medd.*, **1**, No. 12.
—— (1920, 1). *Biochem. J.*, **14**, 267.
—— (1920, 2). *Biochem. J.*, **14**, 282.
—— A. and M. (1913). *Medd. om Grönland*, **51**, 19.
Liljestrand (1916). *Skand. Arch. Physiol.*, **33**, 161.
—— (1917). *Skand. Arch. Physiol.*, **35**.
Lindhard (1915). *Pflüger's Arch.*, **161**, 377.
Loewy (1911). *Oppenheimer's Handb. Biochemie*, **4**, 281.
Zuntz (1901). *Pflüger's Arch.*, **83**, 564.
—— (1911). Die Quellen der Muskelkraft, *Oppenheimer's Handb. Biochemie*, **4**, 826.
—— and Loeb (1894). *Arch. Anat. Physiol.*, 571.
—— and Loewy (1913). *Lehrb. der Physiologie der Menschens*, 663.
—— and Schumburg (1901). *Physiologie des Marsches*, Berlin.

XXXI. STUDIES IN THE ACETONE CONCENTRATION IN BLOOD, URINE, AND ALVEOLAR AIR. II: THE PASSAGE OF ACETONE AND ACETO-ACETIC ACID INTO THE URINE.

By ERIK MATTEO PROCHET WIDMARK.

From the Physiological Institute, Lund, Sweden.

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There is a great difference between the conditions of diffusion in the tissues in the case of the two components of the total acetone, the acetone and the aceto-acetic acid. The aceto-acetic acid occurs within the organism as a largely dissociated salt. Of the salts of the organic acids we know that they possess only to an inconsiderable degree the power of penetrating the living cell. The tissue cells therefore constitute a certain obstacle to the free diffusion of the salt. On this account a difference in concentration may arise and remain on the two sides of a tissue membrane. Acetone on the other hand belongs to a group of substances which can with the greatest ease penetrate the living cell. It can therefore, on its appearance within the organism, diffuse without any hindrance from cells or cell membranes.

This difference appears clearly in the passage of these two substances into the urine.

The aceto-acetic acid is concentrated through the action of the kidneys. The acetone passes into the urine by means of diffusion, and no concentration of the free acetone in the urine can be brought about by the action of the kidneys.

In the urinary bladder the concentration of the aceto-acetates is considerably greater than the concentration of these substances in the blood; diffusion through the walls of the bladder is not probable. The acetone on the other hand is very readily diffused through the walls of the bladder.

In this part we give details of a number of experiments intended to throw light upon these facts.

The elimination of acetone through the urine.

The idea that substances soluble in lipoids pass into the urine solely through diffusion was formulated more than ten years ago by Overton [1907]. He writes: "Wenn Amphibien in nicht tötlichen Konzentrationen von Alkohol, Aceton u.s.w. suspendiert werden, so enthält der Harn nach Ablauf einiger Zeit [die zur Einstellung des Gleichgewichts zwischen dem Gehalte

der Verbindungen im Blute und der Aussenlösung erforderlich ist] diese Stoffe in annähernd gleicher Konzentration wie die Aussenlösung [nach eigenen, noch nicht publizierten Versuchen]. Auch bei den Säugetieren fand Gréhant nach Einführung von Alkohol in den Magen fast die gleiche Alkoholkonzentration im Blute und im Harne. Wegen der schnellen Verbrennung des Alkohols bei warmblütigen Tieren ändert sich die Alkoholkonzentration im Blute recht schnell, und bei dem ungünstigen Verhältnis der absorbierenden Oberfläche der mässig oder stärker gefüllten Harnblase zu deren Volumen [namentlich bei grösseren Tieren] kann sich die Alkoholkonzentration jenes Harnes, der zu einer Zeit abgesondert wurde, wo sich viel Alkohol im Blute befand, nicht mehr ausgleichen mit der Konzentration im Blute, nachdem dieses an Alkohol arm geworden ist. Wenn umgekehrt viel Harn in der Harnblase vorhanden war, ehe die Alkoholkonzentration im Blute ihr Maximum erreicht hatte, muss zu einer bestimmten Zeit die Alkoholkonzentration in der Harnblase hinter derjenigen im Blute stehen. Ähnliches gilt natürlich für andere Vertreter dieser Gruppe von Stoffen.

"Dass viele Verbindungen, die in den Lipoiden leicht löslich sind, im Organismus durch Paarung mit anderen im Körper gebildeten Stoffen oder auf anderen Weise in Substanzen übergehen, die nicht mehr in den Lipoiden merklich löslich sind, und einer aktiven Exkretion durch die Drüsenzelltätigkeit unterliegen, ist einer Sache für sich, die in keinem Widerspruch mit dem vorher Gesagten steht. Solche organische Verbindungen die nur schwer löslich in den Lipoiden sind, wie Glycerin und Harnstoff, sind ebenfalls in gewissen Drüsen der aktiven exkretorischen Zellentätigkeit unterworfen."

No further detailed researches have yet been published by Overton.

The passage of ethyl alcohol into the urine was investigated in detail some years ago by the author [Widmark, 1915]. Comparative estimations were then carried out between the alcohol percentage in the blood and in the urine simultaneously secreted. The results showed that the concentrations in blood and urine very nearly coincided with one another. The alcohol concentration in the urine was not affected by diuresis; an increase in diuresis caused by the taking of a quantity of water did not in any way change the alcohol concentration in the urine.

The agreement between the concentrations in the blood and in the urine has more recently been demonstrated, independently of the author, by Chabanier and Ibarra-Loring [1916], whose experiments were concerned with ethyl and methyl alcohols.

Experimental.

The experiments with acetone were carried out as follows:

The subject first completely emptied his bladder. He then drank a certain quantity of dilute acetone solution. Blood estimations were at once carried out, with a few minutes interval after each. Every 15th minute the bladder was emptied, the quantity of urine measured, and the acetone concentration

determined. In certain experiments this series of estimations began immediately after the taking of acetone; in other cases the comparative estimations were not begun until after the acetone concentration in the blood had become something like constant. In order to demonstrate the influence of diuresis, or more properly speaking to show that diuresis has no influence upon the acetone concentration in the urine, a quantity of water was taken at a certain point in the experiment, the variation in the quantity of urine secreted during the periods of 15 minutes was measured, and the acetone concentration estimated.

Experiment 1. Subject of experiment: the author. Acetone taken: 8 grams in about 200 cc. of water. First blood test 7 minutes after taking, first urination 15 minutes after taking. During the sixth 15-minute period 300 cc. of water at room temperature were taken. The results of the experiment are shown by Table I and Fig. 1.

Table I.

Time (Periods of 15 min.)	Urine: Concentration of acetone ‰	Urine: Rate of excretion cc./min.	Blood: Concentration of acetone ‰
I	0·01	2·3	0·04
II	0·12	2·8	0·15
III	0·16	9·2	0·12
IV	0·16	10·7	0·12
V	0·14	4·5	0·12
VI	0·14	3·2	0·12
VII	0·14	5·7	0·11
VIII	0·13	10·7	0·12
IX	0·14	3·6	0·12
X	0·13	3·7	0·10
XVII	0·10	2·9	0·08

The table and figure show that the acetone concentration of the blood at first rises more rapidly than that of the urine; the highest values in the blood were obtained sooner than the highest values in the urine. The connection between the two cannot be ascertained more closely, because the periods during which the urine was collected in the bladder are too long for the estimation of the concentration in the portions obtained to express accurately the course of the curve. When the blood acetone has shown itself to be at a certain fairly constant level, the urine acetone also remains fairly constant, as far as the concentration is concerned. The concentration of acetone in the urine remains throughout somewhat higher than that in the blood. This is probably due to the fact that the acetone in the urine has about the same concentration as that in the plasma, which is somewhat higher than that of the total blood. Possibly the concentration in the urine may be even a little higher than in the plasma, on account of the fact that acetone is perhaps more easily soluble in urine than in plasma. The saltness of the urine does not seem to play any part worth mentioning. During the experiment the excretion curve reached two maxima: the first due to the

quantity of fluid taken in connection with the taking of the acetone, the second to the quantity of water taken during the sixth period. The concentration in the urine in no way follows the variations in the excretion, but seems to proceed in complete independence of these.

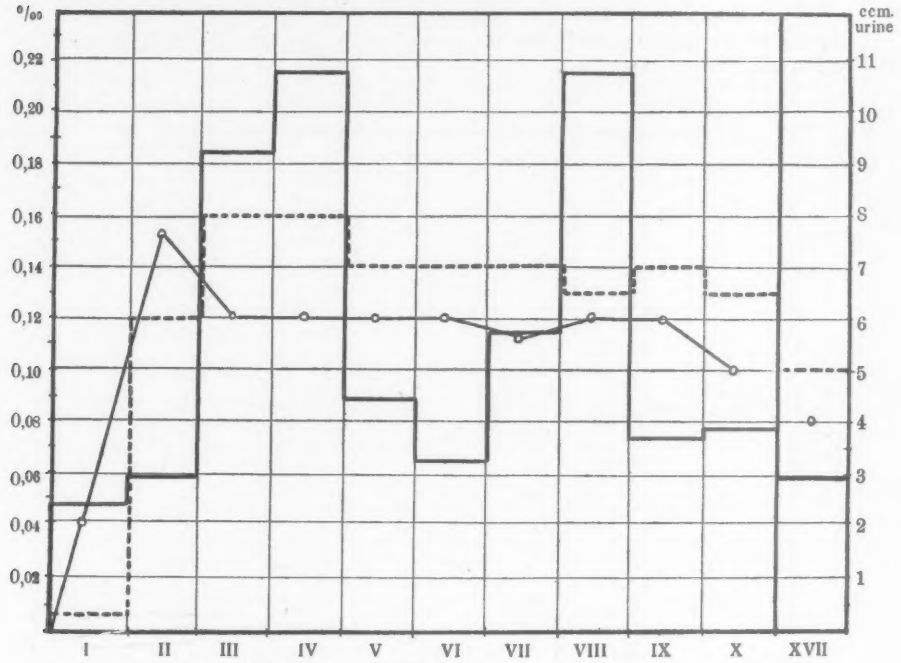

Fig. 1. ——=excretion.
- - - -=concentration of acetone in urine.
o—o=concentration of acetone in blood.
Abscissa: periods of analysis.
Ordinate: acetone concentration in ‰, excretion in cc. per min.

Experiment 2. Subject: the author. On March 16 at 10.45 a.m., 9·6 g. of acetone were taken in 500 cc. of water. The comparative estimations of the acetone concentration in blood and urine were begun at 1.15 p.m. At 1.45 p.m. 500 cc. of water were taken. The result is shown by Table II and Fig. 2.

Table II.

	Urine		Blood	
Time	Concentration of acetone ‰	Rate of excretion cc./min.	Time	Concentration of acetone ‰
1.15–1.30	0·160	1·33	1.35	0·127
1.30–1.45	0·160	1·80	2.20	0·127
1.45–2.00	0·160	2·13	2.25	0·124
2.00–2.15	0·160	8·40	2.50	0·115
2.15–2.30	0·160	7·33	—	—
2.30–2.45	0·156	2·87	—	—

This experiment shows, even better than the preceding, how completely the acetone concentration of the urine is independent of the amount excreted. Here too the concentration of acetone in the urine is somewhat higher than in the blood. The lowest value for the rate of excretion is 1·33, the highest is 8·4; although therefore the volume of the urine becomes more than six times as great, the concentration of the acetone is not in the slightest degree influenced by this fact.

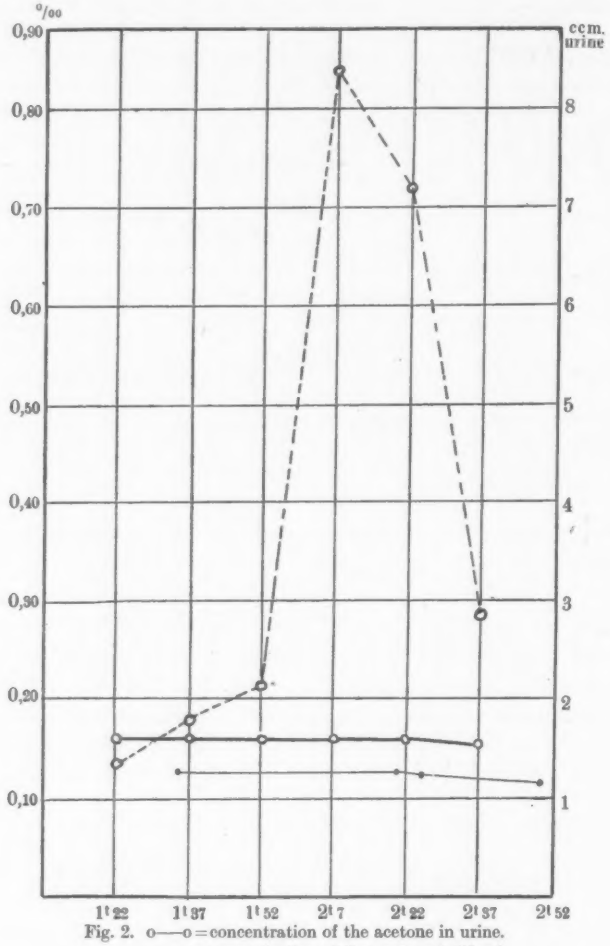

Fig. 2. o—o = concentration of the acetone in urine.
——— = concentration of the acetone in blood.
o- - -o = excretion.

In this graph, for the sake of clearness, both the excretion and the estimations of the acetone concentration of the urine are represented by points lying in the middle of the period in question.

Experiment 3. Subject: the author. On March 7, 1917 at 9.45 a.m., 16 g. of acetone were taken in 600 cc. of water. The comparison between the acetone concentration in blood and urine was begun at 1.15 p.m. At 2.35 p.m. 500 cc. of water were taken.

Table III.

	Urine		Blood	
Time	Concentration of acetone °/₀₀	Rate of excretion cc./min.	Time	Concentration of acetone °/₀₀
1.15–1.30	0·28	1·87	1.15	0·22
1.30–1.45	—	1·67	—	—
1.45–2.00	0·29	1·80	—	—
2.00–2.15	—	1·87	—	—
2.15–2.30	0·27	1·67	—	—
2.30–2.45	0·27	1·87	—	—
2.45–3.00	0·25	3·73	—	—
3.00–3.15	0·26	9·67	3.14	0·21
3.15–3.30	0·25	10·53	—	—
3.30–3.45	0·23	5·73	4.0	0·18

Experiment 4. Subject: B. J. Body weight, 63 kg. At 11.40 a.m. 8 g. of acetone were taken in 500 cc. of water. The comparison between the acetone concentration in blood and in urine was begun at 2.30 p.m. At 2.35 p.m. 500 cc. of water were taken.

Table IV.

	Urine		Blood	
Time	Concentration of acetone °/₀₀	Rate of excretion cc./min.	Time	Concentration of acetone °/₀₀
2.30–2.45	0·15	1·60	2.12	0·15
2.45–3.00	0·16	4·93	3.15	0·13
3.00–3.15	0·17	8·80	4.12	0·12
3.15–3.30	0·16	8·40	—	—
3.30–3.45	0·17	8·67	—	—

This experiment, carried out on a different subject from the preceding, shows the same facts as have been already described.

The elimination of aceto-acetic acid through the kidneys.

If an experiment in the same direction as those recorded above is made with a diabetic patient with acidosis—*i.e.* if he is allowed to drink a certain quantity of water, and comparative measurements are then taken of the total acetone concentration in the blood and in the quantity of urine passed during each 15-minute period—quite a different result is obtained. We know that by far the greatest part of the total acetone of the urine occurs in the form of aceto-acetates. Only a small part is free acetone. It has already been pointed out that the conditions of diffusion for these salts are quite other than for the acetone. The salts are not diffused through the kidneys but may evidently be subjected to an active excretion, through which the concentration of these substances in the urine may become many times greater than their

concentration in the blood. With an increased quantity of urine resulting from the resorption of a certain amount of water by the subject, the amount of the aceto-acetates secreted is not increased in the same proportion as the amount of urine. The consequence of this is that with a water-diuresis the total acetone concentration sinks rapidly, to rise again later as soon as the rate of excretion of urine returns to its normal value. This is shown by the two following experiments.

Experiment 5. August 16, 1916. Subject: Mrs L., diabetic with pronounced acidosis. 1 litre of water was taken on an empty stomach. The total acetone concentration was then estimated in portions of urine obtained every quarter of an hour. The estimations were carried out according to the method of Embden and Schliep. The reaction of the urine was alkaline or amphoteric.

Table V.

Time (Periods of 15 min.)	Total acetone concentration of urine °/₀₀	Rate of excretion of urine cc./min.
I	0·696	0·53
II	0·658	0·60
III	0·482	0·93
IV	0·189	2·73
V	0·193	3·60
VI	0·244	1·80
VII	0·310	1·67

As soon as the rate of excretion is increased, the total acetone concentration in the urine simultaneously falls to a considerable extent. After the rate has reached its maximum and has begun to sink, the total acetone concentration rises.

Experiment 6. Subject: J. N., diabetic with acidosis. At 1.45 p.m. on March 14, 1917, one hour after a meal, 500 cc. of water at room temperature were taken. The reaction of the urine was alkaline or neutral. The results are given in Table VI and Fig. 3.

Table VI.

	Urine		Blood
Time	Total acetone °/₀₀	Rate of excretion cc./min.	Total acetone °/₀₀
1.45–2.00	—	0·60	—
2.00–2.15	> 0·95	0·86	2.20 {0·109 / 0·103
2.15–2.30	0·31	4·26	—
2.30–2.45	0·19	7·73	—
2.45–3.00	0·25	6·40	3.20 {0·126 / 0·121
3.00–3.15	0·72	1·60	—

This experiment shows that the total acetone concentration in the urine is throughout considerably higher than in the blood. When the diuresis sets

in the concentration in the urine at once sinks to a considerable extent, and rises again as soon as the quantity of urine is diminished. There is therefore no correlation between the acetone curves in the blood and in the urine, and the passage of the aceto-acetic acid into the urine is determined by the incidental secretive activity of the kidneys[1].

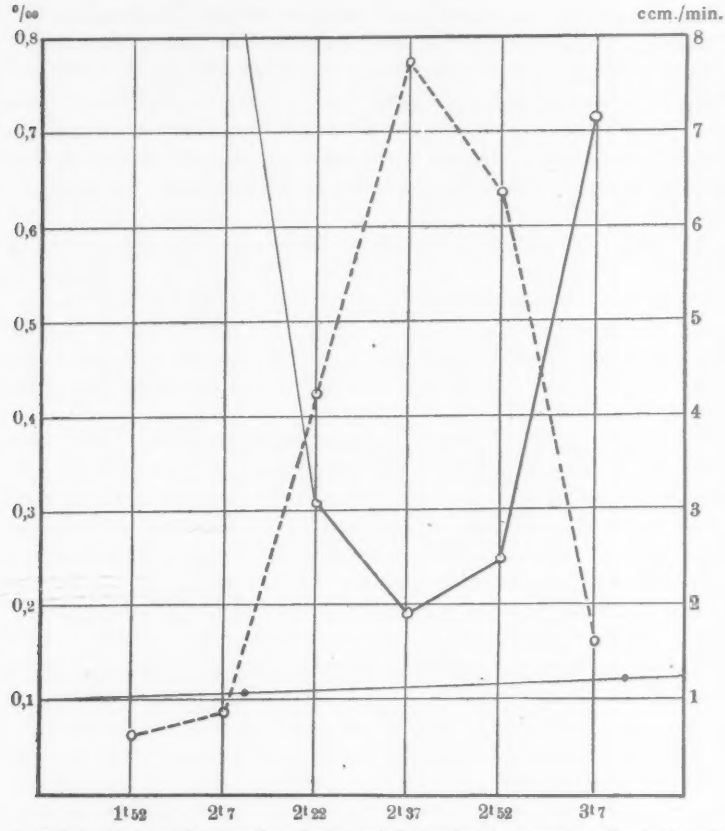

Fig. 3. Relation between the excretion of urine and the total acetone concentration in the urine in diabetes, according to Exp. 6. o- -o = excretion of urine; o—o = total acetone concentration in urine; —— = total acetone concentration in blood.

[1] It is possible that the *reaction* of the urine may also play a certain part in the elimination of aceto-acetic acid. The free acid may possibly be more easily diffused than the salt through the kidney epithelium; a difference in concentration will in that case less easily arise. In this circumstance may perhaps lie the explanation of the fact which many writers claim to have demonstrated, that the acetone bodies pass over into the urine in greater quantities if the patient is given bicarbonate.

On the relation of the acetone to the walls of the urinary bladder.

It was formerly believed that the walls of the healthy urinary bladder are, practically speaking, impermeable to the substances which pass into the bladder by way of the kidneys, and that these substances can only leave the bladder by one way, viz. through the urethra [compare Cohnheim, 1909].

Novicka [1913] has however quite incontestably shown that alcohol is diffused with great ease through the walls of the bladder, the direction of diffusion depending upon the higher concentration. For this substance therefore the rule does not always apply that the amount of alcohol found in the daily quantity of urine is also the daily quantity of alcohol coming from the kidneys. The following cases, for instance, may be imagined to occur:

(*a*) The alcohol concentration in the bladder and the blood is the same during the period between two urinations. The quantity of alcohol which has come from the kidneys is not altered in the bladder by diffusion.

(*b*) The alcohol concentration is lower in the blood than in the bladder during the time that elapses between two urinations. The quantity of alcohol which has come from the kidneys is diminished in the bladder by diffusion from bladder to blood.

(*c*) The alcohol concentration is higher in the blood than in the bladder during the period between two urinations. The quantity of alcohol which has come from the kidneys is increased by direct diffusion from blood to bladder.

If the time between the urinations is short, the difference in concentration between blood and urine will be inconsiderable, because the alcohol, as we have previously emphasised, passes into the urine in the same concentration as into the blood, and no considerable fluctuations in concentration can appear. If the time between two urinations is long, it may happen that the alterations in the concentration of the blood are of so high a degree that cases (*b*) and (*c*) may occur.

It is evident that this property possessed by ethyl alcohol of diffusion through the walls of the bladder is merely an evidence of its power to penetrate the living cell and organic tissues in general. It is therefore to be expected that all substances having the same properties as alcohol will form an exception to the rule as to the impermeability of the walls of the bladder[1].

In the following we shall show that the relation of acetone to the walls of the bladder is exactly the same as that of alcohol.

[1] Thus, for example, methylene blue diffuses with the greatest ease through the bladder walls. If the ureters of a rabbit are bound up and a dilute solution of methylene blue is injected into vena jugularis, it will be found very soon—18 minutes after the injection—that the urine is coloured deep blue.

Demonstration of the diffusion of acetone from bladder to blood.

Experiment 7. August 24, 1916. Rabbit ♂. A half-solid catheter was introduced into the bladder and allowed to remain throughout the experiment. 45 cc. of urine were drawn off. 50·5 cc. of 2·42 °/₀₀ acetone in physiological salt-solution—*i.e.* 122 mg. of acetone—were then introduced. After one hour the bladder was tapped and rinsed out three times with 5 cc. of physiological salt-solution. In the mixed quantity the acetone was determined according to Messinger-Huppert and the amount found to be 71·4 mg.

Result:		
	Introduced	122 mg.
	Found	71 mg.
	Quantity lacking after one hour	51 mg. = 41·8 %

The blood contained 1·54 mg. of acetone per 100 cc. Post mortem examination showed an intact bladder mucous membrane. Otherwise nothing pathological.

Experiment 8. March 5, 1917. Rabbit 1330 g. ♂. At 11.10 a.m. a few cc. of a 5 % acetone solution were injected by means of a catheter into the animal's bladder. A series of blood estimations was then undertaken. The result is shown by Table VII.

Table VII. The passage of acetone from the contents of a rabbit's bladder into the blood.

Time	Total acetone of blood °/₀₀
11.0 a.m. before the injection	0·010, 0·012 = 0·010
12.15 p.m.	0·10
1.25	0·11
2.25	0·13
3.20	0·09

The experiment shows that acetone introduced into the bladder can be demonstrated in the blood as soon as one hour afterwards.

Even though it is highly probable that there is no important dissimilarity between the conditions of diffusion in the urinary bladder of rabbits and of human beings, the demonstration of the diffusion in human beings is nevertheless of importance. For the introduction of a definite amount of acetone into the bladder of human beings a modification of Janet's method is used. The arrangement of the apparatus is shown by Fig. 4. *A* is a vessel that can be raised and lowered, attached by a tube to the pressure-flask *B*, communicating with the burette *C*, which has a glass tap at either end. The discharging-tube of the burette is provided with a perforated rubber cone, which can be introduced into fossa navicularis penis so as to close the orifice of the urethra. At the beginning of the experiment all the vessels contain sublimate solution (1 : 2000). By lowering *A* beneath the level of *B* and giving *C* a suitable position with the fluid above the tap *b*, the sublimate

solution can be conveniently sucked out of the burette and sterile acetone solution sucked in up to a certain height. When the pressure has been equalised by raising *A*, the taps are closed, and the level in the burette is read off. The burette is now brought into contact with the urethra, the taps are opened once more, and *A* is gently raised to a suitable height. When a sufficient quantity of fluid has passed into the bladder, *A* is lowered and the amount of fluid present in the distended urethra flows back into the burette, with the exception of a very small quantity (0·5–1·0 cc.), which can be caught and measured when the burette is removed from the penis. Before this is

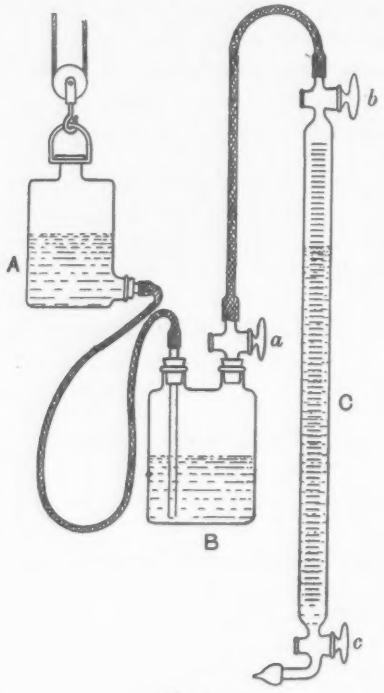

Fig. 4.

done the taps are closed and the burette is read off. By this arrangement an accurately measured quantity of fluid can be introduced into the bladder without pain or any appreciable discomfort. The fluid in the burette can be kept at body temperature. After the injection the concentration of the acetone in the burette is determined. The burette has all the time been completely closed, except during the injection. This removes the possibility of an error which might arise owing to the evaporation of the acetone solution from an open vessel.

Experiment 9. Subject: E. W. August 25, 1916. 37 cc. of a 1 % solution of boracic acid containing 0·74 °/₀₀ acetone were introduced into the bladder. An hour after this the bladder was emptied. In order to wash out as much as possible of the acetone remaining, the bladder was emptied a couple of times more, with 5–10 minutes' interval between. The total quantity of urine amounted to 294 cc. It contained 22·8 mg. of acetone.

Result:			
	Introduced	27·4 mg. acetone	
	Found	22·8 „	
	Amount lacking after one hour	4·6 „	=16·8 %

This experiment suffers from two deficiencies: (1) the amount of acetone introduced is too small, and (2) the experiment was carried out immediately after a meal, so that the excretion of urine was comparatively great. For both these reasons the pre-formed iodine-binding substances present in the urine give rise to a relatively great source of fallacy. The result of the experiment may therefore easily lead to the conclusion that a less complete reabsorption takes place than is actually the case. The following experiment, in which a larger quantity of acetone was introduced during a period of low rate of excretion, gives a clearer idea of the diffusion.

Experiment 10. Subject: E. W. August 26, 1916. 57 cc. of a 2·77 °/₀₀ acetone solution were introduced. After one hour the bladder was emptied as in the previous experiment. The total quantity of urine, 178 cc., contained 114 mg. of acetone.

Result:			
	Introduced	158 mg.	
	Found	114 mg.	
	Amount lacking after one hour	44 mg.	=27·8 %

The experiments show that acetone is able to pass quite easily through the walls of the human bladder also. It should however be pointed out that a certain quantity of acetone may have diffused into the blood in the passage through the urethra during the introduction of the liquid. The walls of the ureters are of course permeable to a far higher degree, for many substances, than the walls of the bladder. Nevertheless, that there is a diffusion through the walls of the bladder can hardly be denied.

Demonstration of the diffusion of acetone from blood to urine with tied ureters.

Experiment 11. August 23, 1916. Rabbit ♂. Weight 2640 g. Narcosis with ethylene urethane. After ventral incision in the middle line both ureters were brought forward and tied immediately above the bladder. The wound was provisionally closed. A half-solid catheter was then introduced through the urethra and 46 cc. of urine were drawn off. 50 cc. of physiological salt-solution were introduced.

Now, for a period of ten minutes, 15 cc. of about 50 % acetone in physiological salt-solution were injected drop by drop through the vena jugularis

externa. The rabbit showed strong dyspnoea during the injection. Five minutes after the injection was over Legal's acetone test on 2 cc. of the contents of the bladder showed uncertain reaction. After five minutes more the test was clearly positive, and 15 minutes after the injection strongly positive: 30 minutes after the injection a blood sample was taken and the urine left in the bladder drawn off. Acetone estimations on both samples according to Messinger-Huppert showed:

In the blood: 3·2 ‰ acetone.
In the contents of the bladder: 1·1 ‰ „

Section showed an intact bladder mucous membrane. In other respects nothing noteworthy.

These experiments show therefore that free acetone can pass with considerable ease through the walls of the urinary bladder. The direction is determined by the side of the bladder walls on which the greater concentration prevails. The quantity diffused will depend on the concentration and the extent of the absorbing surface.

In diabetes the changes in the percentage of free acetone in the blood probably take place somewhat slowly. In all probability no considerable difference in concentration occurs between the free acetone of the blood and that of the bladder, nor, consequently, is there any considerable change in the amount of acetone in the urine owing to diffusion through the walls of the bladder. On the other hand it is conceivable, especially if the urine is strongly acid, that part of the aceto-acetic acid is already decomposed in the bladder. On account of the higher acetone concentration which thus appears in the bladder, a diffusion of acetone into the blood may take place. The extent of the decomposition of the aceto-acetic acid under such circumstances is not known. No kinetic measurements of the decomposition of the acid under different circumstances are yet forthcoming. We cannot decide, therefore, how great a diminution is undergone by the amount of aceto-acetic acid secreted by the kidneys as a result of the fact that part of it is already decomposed in the bladder and the acetone arising therefrom diffused through the bladder walls.

I have performed no experiments with regard to the relation of aceto-acetic acid to the walls of the urinary bladder. From the fact that the total acetone concentration of the urine is usually higher than that of the blood we may conclude that diffusion through the walls of the bladder cannot take place to any considerable extent.

Formula for the elimination of acetone through the kidneys.

Free acetone is eliminated—as soon as more violent variations in the acetone concentration of the blood have ceased to take place—in a concentration which lies very near to that of the acetone concentration in the plasma. This concentration remains unchanged even though the amount of urine excreted during the unit of time varies.

The amount of acetone, M_u, coming from the kidneys in the unit of time depends therefore upon the following three factors:

The concentration of free acetone in the plasma, a,

The partition coefficient urine/plasma, κ,

The rate of excretion, quantity of urine per unit of time, v,

or $$M_u = a.v.\kappa.$$

If free acetone exclusively is present in the blood, the quantity eliminated may be approximately estimated from the above factors. With constant blood acetone concentration the amount of acetone eliminated will be increased proportionally to the increase in the quantity of urine. If this quantity of urine is increased to twice as much, *e.g.* because the subject of the experiment drinks a certain quantity of water, the amount of acetone eliminated will also be doubled. With a constant quantity of urine the amount of acetone eliminated increases proportionally to the increase in the concentration in the blood, provided that this increase does not proceed too quickly.

κ for urine/plasma is in all probability very nearly 1. For urine/total blood—according to calculation from the values given by the published experiments—it is 1·235, with $E = \pm 0{\cdot}0670$ and $e_1 = \pm 0{\cdot}0186$.

If, as well as the free acetone, aceto-acetic acid simultaneously occurs, the total quantity of acetone eliminated through the urine cannot be calculated in the same simple fashion. The amount of aceto-acetic acid secreted depends not only on the aceto-acetic acid concentration in the blood but also on the capacity of the kidneys, a factor which cannot at present be determined. The formula for the elimination of the total acetone, M_t, in the unit of time may (for a purpose to be mentioned later) be written:

$$M_t = a.v.\kappa + c,$$

where c is unknown.

By throwing light on this proposition we shall be able to arrive at some explanation of the relations between the acetone and the aceto-acetic acid in the urine.

As is well known, Folin [1907] and Embden [1907] have found that the pre-formed acetone in the urine generally constitutes a quite inconsiderable proportion of the total acetone of the urine. From Folin's measurements we take the following table in which all the values are reckoned in °/₀₀.

Table VIII. Some of Folin's estimations concerning the relation between acetone and aceto-acetic acid in the urine of diabetics. All the values are expressed in °/₀₀.

No.	Total acetone	Free acetone	Acetone from aceto-acetic acid
I	0·67	0·16	0·51
II	0·68	0·25	0·43
III	2·83	0·36	2·47
IV	0·79	0·24	0·55
V	2·15	0·26	1·89
VI	1·67	0·22	1·45
VII	1·02	0·10	0·92

Embden finds that often only aceto-acetic acid occurs in the urine. In general the amount of acetone in his estimations constituted less than 25 % of the total acetone, and most frequently considerably under 10 %.

Now we know that the free acetone—provided that no very considerable decomposition of the aceto-acetic acid takes place in the bladder—can never reach values lying appreciably higher than the concentration of the free acetone in the blood. It is only in very rare cases that the total acetone in the blood of diabetics is as much as 0·40 ‰; the free acetone therefore has still lower values. Hence it follows that the acetone in the urine can never reach any very high concentration. But the aceto-acetic acid, which is secreted by the kidneys, may on the other hand be present in the urine in a concentration many times greater than that of the blood.

Summary.

Acetone passes into the urine by way of diffusion. The concentration in the blood and in the urine is therefore usually the same.

Aceto-acetic acid passes into the urine by way of the concentrating activities of the kidneys. The concentration in the urine is therefore higher than in the blood.

REFERENCES.

Chabanier and Ibarra-Loring (1916). *Compt. Rend. Soc. Biol.*, **79**, 8.
Cohnheim (1909). Zuntz and Loewy's *Physiologie des Menschen*, p. 588.
Embden (1907). *Verhandl. Kongr. f. inn. Med.*, **24**, 252.
Folin (1907). *J. Biol. Chem.*, **3**, 177.
Novicka (1913). *Contribution à l'étude de la perméabilité et du pouvoir absorbant de la vessie*, Thèse, Paris.
Overton (1907). "Ueber den Mekanismus der Resorption und der Sekretion," Nagel's *Handbuch d. Physiol.*, **2**, 744.
Widmark (1915). *Skand. Arch. Physiol.*, **33**, 55.

XXXII. STUDIES IN THE ACETONE CONCENTRATION IN BLOOD, URINE, AND ALVEOLAR AIR. III: THE ELIMINATION OF ACETONE THROUGH THE LUNGS.

By ERIK MATTEO PROCHET WIDMARK.

From the Physiological Institute, Lund, Sweden.

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In the following an attempt is made at an exact analysis of the passage of acetone into the expiratory air, and in so doing I have tried in the main to follow the method of analysis adopted in modern experiments in regard to the exchange of respiratory gases[1].

If the passage of acetone into the alveolar air is a pure diffusion process, the partial pressure of the acetone vapour in the latter should never be higher than the partial pressure in the blood. But if, on the contrary, an energetic, secretory activity is contributed by the lungs, the partial pressure of the acetone in the alveolar air may be higher than in the blood. The decision of these points becomes much more simple in the case of acetone than in the case of the respiratory gases. The latter are partly dissolved and partly in chemical combination in the blood, and their concentrations are subjected to great variations in the venous and the arterial blood. The acetone, on the other hand, is exclusively dissolved in the blood, and the concentration remains pretty nearly constant for hours [Widmark, 1919]. Certainly after resorption no demonstrable difference in concentration occurs in the arterial and venous blood.

In the physiology of respiration the absorption coefficient, as defined by Bunsen [1877], has been used in the estimation of the pressure of the gases in blood and air. The custom of thus expressing by the absorption coefficient the partition of a gas between a fluid and a gas above the fluid is associated with the fact that the gas analyses have chiefly been carried out by the eudiometric method.

The absorption coefficient may evidently be regarded as a partition coefficient for the gas in question with reference to a fluid and a gaseous phase, respect being taken to the particular pressure and temperature of the gases. If we estimate in the direct chemical way *the concentration* of a substance which is divided between a gaseous and a fluid medium it is to a certain

[1] No historical review is given in this paper The reader is referred to my book [1917].

extent a roundabout method to express the division by the absorption coefficient. It is simpler to express it in the same way as when we are dealing with the distribution of a substance between two fluid media. Here the concentration of the substance in the gaseous phase must naturally be calculated upon the volume which the gas has when diffusion equilibrium is attained.

As far as acetone is concerned, therefore, we need not express the concentration in partial pressure; it is simplest to give the quantity by weight of acetone per cc. of air and per cc. of fluid. The coefficient of partition (λ) therefore takes the following expression:

$$\lambda = \frac{[\text{acetone in air}]}{[\text{acetone in fluid}]}.$$

This method of expression is made use of in the following pages. Naturally the values can easily be calculated again in mm. of mercury-pressure. In that case we should have to do with fractions of a mm., although the exposition would not gain in clearness thereby.

The above method of expression can naturally only be used when the partial pressure of the binary fluid complex is directly proportional to the dilution. It is not deducible *a priori*—nor indeed is it probable—that such a simple state of affairs exists, in regard to acetone, throughout the whole dilution from 100–0 %.

For our purpose, however, it is only of interest to know the coefficient of partition between air and fluid within the domain represented by the acetone concentration in the fluid from 0–1 °/₀₀. This is consequently only a thousandth part of the whole curve of partition, and in regard to this part we may assume that it will be approximately straight, *i.e.* that a constant relationship will exist between the acetone concentration in fluid and in air. This is also shown by the experiments.

The partial pressure in the case of the system water-acetone has previously been studied by Carveth [1899]. He has determined the composition of the vapour from this binary fluid and has calculated the partial pressure of the acetone. His measurements however cannot be used for the particular relationships in question. It is therefore necessary to elucidate these experimentally.

Our first object then will be to determine the partition of acetone between air and fluid for solutions under 1 °/₀₀. Further, in order that it may be possible to apply the measurements to physiological conditions, the coefficient of partition should be determined for a temperature of 38°.

The determination of the coefficient of partition has been carried out in two different ways, first by determining the quantity of acetone which an acetone solution loses when shaken with a certain volume of air at 38° (method *A*), and secondly by an analysis of air shaken at the same temperature with acetone solution of known strength (method *B*).

After determining λ for water, blood, and serum I have proceeded to examine whether the acetone concentration in alveolar air shows a value that is in agreement with this coefficient.

A. Determination of λ by estimations of the concentration of the acetone solution before and after shaking with a definite quantity of air.

For these determinations a litre flask was used, the neck of which was drawn out into a glass tube about 13 cm. long and 5 mm. in diameter. Over the tube was passed a glass cup which was fixed to the lower end of the tube by a piece of rubber tubing. The cup was filled with mercury up to a certain mark. An inverted test-tube was fitted over the glass tube, its opening reaching down into the mercury. The test-tube was supported by the bottom of the cup and was further fixed by means of a perforated rubber stopper passing over the test-tube and fitting into the orifice of the cup, so that this became perfectly air-tight. The space thus enclosed was accurately measured with water and found to contain 1230 cc.

The flask was shaken in a machine driven by a motor. It was enclosed in a case which could be kept at a constant temperature of 38°, at which the shaking was performed. The temperature was regulated by a spirally twisted bimetallic spring which changed its curvature with variations in temperature, and according to the movements of this an electric current was shut off or opened, this current in its turn affecting an electro-magnetic key which regulated the flow of gas to a burner placed inside the case. The temperature was regulated to about 0·1°. The apparatus was constructed at the Lund Physiological Institute.

The experiments were carried out as follows. In the flask was placed an accurately measured quantity of acetone solution, usually 12 cc., in some cases 3 cc. The flask was then closed, placed in the shaking-machine, and shaken for one hour at 38°. A test experiment showed that this period was long enough to admit of the establishment of a balance between the acetone concentration in the air and in the fluid. After the shaking the flask was taken out of the machine, the mercury in the cup poured off, and the flask turned upside down so that the acetone solution might run down quickly into the test-tube. Samples for analysis were then taken from the latter. In experiments with pure aqueous solutions the analysis was performed according to Messinger; in experiments with blood and serum the estimation was carried out after distillation in the usual way, either by the macro- or the micro-method.

Full details are here given of one experiment. The other estimations were carried out in exactly the same way.

Experiment 1. February 27, 1917. In the flask was placed 12 cc. of 0·809 $^{o}/_{oo}$ acetone solution. After one hour's shaking at 38° the solution was

found to be 0·648 °/₀₀. By heating from 20–37° the volume of the solution was diminished through evaporation of water from 12 cc. to 11·965 cc. (calculated according to *Chemiker-Kalender*, II, 1917, Table 88).

Amount of acetone introduced	$12 \times 809 = 9708\,\gamma$
Found in the solution after shaking	$11{\cdot}965 \times 648 = 7753\,\gamma$
Amount of acetone evaporated	$1955\,\gamma$

The volume of the flask was 1230 cc. After the introduction of 12 cc. of fluid, therefore, the volume of air over the fluid became $1230 - 12 = 1218$ cc. The quantity of acetone per cc. of air is therefore $\frac{1955}{1218} = 1{\cdot}605\,\gamma$.

From this we calculate

$$\lambda = \frac{1{\cdot}605}{648} = 0{\cdot}00248.$$

In addition to this estimation two further estimations were carried out on pure acetone solutions. These were performed exactly as has just been described. In the table given below all the estimations will be found set out together.

Table I. Summary of estimations of the partition of acetone between air and water. The second column contains the results of the analysis of the acetone solution after the shaking, the third column gives the calculated quantity of acetone per cc. of air, and the fourth the values of λ.

	Acetone found in solution after shaking γ/cc.	Acetone in air (by difference) γ/cc.	λ
1	648	1·61	0·00248
2	646	1·62	0·00251
3	342	0·82	0·00240
			Average: 0·00246

The coefficient of partition for acetone between water and air at 38° is therefore, according to these experiments, 0·00246.

The coefficient of partition *between serum and air* ought presumably to be somewhat higher than for pure aqueous solutions, on account of the saltness of the serum. An estimation however showed no certain difference.

Experiment 2. For this estimation a quantity of defibrinated calves' blood was centrifuged, the serum pipetted off, and a small quantity of pure acetone added. Into the flask was introduced 3 cc. of serum, which was shaken for one hour at 38°. The acetone concentration was determined in 0·2 cc. by the micro-method, and was

Before shaking °/₀₀	After shaking °/₀₀
0·392	0·199
0·387	0·184
0·378	0·187
Average: 0·386	0·190

This gives $\lambda = 0{\cdot}00255$.

In the determination of the coefficient of partition between *air and total blood* higher values were obtained for λ.

Experiment 3. 15 cc. of defibrinated cow's blood was shaken for one hour at 38°. The determination of the acetone concentrations was carried out by the macro-method. The following values were obtained:

Acetone concentration before shaking, 0·826 and 0·823, average 0·825 $^{o}/_{oo}$.

Acetone concentration after shaking, 0·661 $^{o}/_{oo}$.

Hence we get for the acetone concentration in the air, 2·04 γ/cc., and for λ 0·00309. Another measurement gave 0·00329.

The measurement of the coefficient of partition between total blood and air therefore gives a value considerably higher than those previously found. This is easily understood if we assume that the acetone concentration in the blood corpuscles is lower than in serum—an assumption the probability of which is strengthened by other experiments. The total blood should, theoretically speaking, yield the same amount of acetone per cc. of air as the plasma obtained after centrifugalisation. But on the other hand the partition coefficient comes to be somewhat higher for the total blood, since the determination of the acetone concentration in the total blood gives a lower value than in serum.

B. Determination of λ by an analysis of air shaken with acetone solution.

From the experiments just described it follows that the proportion between the amount of acetone per cc. of air and per cc. of solution is about 3: 1000. In 100 cc. of air we ought therefore to find about three times as much acetone as in 0·1 cc. of solution. Consequently there should be quite sufficient quantities of acetone in the air for an estimation by the micro-method to be possible, and the error can therefore be actually three times as great as in the blood estimations without making the error per cent. any greater than in these.

The analysis of air shaken with acetone solution was carried out as follows. In a tube of about 120 cc. capacity, provided with glass taps at both ends, were placed about 50 cc. of acetone solution of known concentration. The tube was then shaken in the shaking-machine for 1½ hours at 38°. A test experiment showed that after one hour's shaking equilibrium had already been set up between the acetone concentration in the air and in the fluid. A couple of times during the shaking process one of the taps of the tube was opened in order to equalise the pressure, which had been increased by the heating from room temperature to 38°. After the shaking the tube was taken out and placed in a small chamber heated by a gas flame to 38°, the temperature of which was kept constant by means of a regulating arrangement constructed like that of the shaking-machine. The air in the tube was here passed over into a Hempel gas burette, provided with correction-tube and manometer. Acetone solution of the same strength as that used in the

shaking served as fluid in the pipette. When the amount of air had been measured at the temperature and pressure which prevailed during the shaking, the air was passed over into three test-tubes, arranged like a series of wash-bottles. The glass tubes, which reached down to the bottom of the test-tubes, were drawn out into fine capillary points. Each of the test-tubes contained 3 cc. of $N/2$ sodium hydroxide + 2 cc. of $N/200$ iodine solution (or 2 cc. of $N/100$ iodine solution in the estimation of greater quantities of acetone). The air was passed very slowly through the solutions in the three test-tubes bubble by bubble.

The titre proved to be diminished when 100 cc. of air from the laboratory had been passed through. In the first tube this diminution amounted to about 0·04 cc. of $N/200$ iodine solution; in the two others it was somewhat less, but still quite perceptible. The change of titre was determined before every series of experiments, and the results of titration were corrected by the values thus obtained from the blind tests.

The acetone was absorbed almost completely by the iodine solution of the first test-tube. Into the second 5–10 % usually passed over, in the third the formation of iodoform could not as a rule be demonstrated with certainty.

The acetone solution used—50 cc.—had in proportion to the volume of air so great a volume that no change in the concentration of the solution after shaking could be observed by titrating. In the calculation I have therefore used the original concentration of the acetone solution.

As an example of the method the following report is adduced.

Experiment 4. January 26, 1917. 50 cc. of 0·164 ‰ acetone were introduced into the shaking tube and shaken for 1½ hours at 38°. The volume of the air analysed was 57·2 cc. and it contained 24·5 γ acetone, *i.e.* per cc.

$$\frac{24{\cdot}5}{57{\cdot}2} = 0{\cdot}43\ \gamma \text{ acetone.}$$

Hence
$$\lambda = \frac{0{\cdot}43}{164} = 0{\cdot}00262.$$

In this way 27 estimations were carried out, in which the strength of the acetone solution varied from 0·164 to 0·822 ‰. Details of these estimations are given in the following table.

Table II. Determinations of the partition coefficient of acetone between air and water at 38°.

Acetone per cc. solution	Acetone per cc. air						Average acetone per cc. air	
γ	γ						γ	λ
164	0·43	0·46	0·44	0·40	0·43	0·39	0·425	0·0026
328	0·94	1·01	0·93	0·99	0·80	0·68	0·891	0·0027
369	0·98	1·11	0·92	0·87	1·03	0·87	0·963	0·0026
576	1·31	1·49	1·30	1·61	1·34	1·21	1·377	0·0024
822	2·15	1·87	2·14	—	—	—	2·053	0·0025

The weighted mean of these values for λ is 0·00257 ± 0·000055. The values show no rise or fall in the case of these solutions, therefore a constant relationship may be considered to exist between the concentration in the air

and in the solution. Fig. 1 (p. 387) gives a graphic representation of the position of the different values in regard to the mean. The abscissa represents the concentration in the aqueous solution expressed in γ/cc., the ordinate the concentration in the air in γ/cc. The values obtained are represented by crosses. The circles represent the values obtained according to method *A*. The straight line is drawn in accordance with the weighted mean value for λ in method *B*. The error is somewhat considerable: $\pm$ 0·00028. This is probably due to the fact that it is very difficult to determine satisfactorily the titre of the blind tests. It has been found also that both rubber tubes and greased glass taps have an extremely injurious effect upon the determinations and cause the values to become irregular; possibly rubber tubes and grease give off small quantities of iodine-binding substances or absorb the acetone. The determination of the mean value is however of sufficient accuracy to admit of the use of this value in the investigation which follows. The values obtained by this process are somewhat higher than those given by the method previously described; the difference however is unimportant.

C. Estimation of acetone in the alveolar air.

If the elimination of acetone through the lungs is a pure diffusion-process we should find—provided that a complete balance of diffusion can be set up—in determinations of the acetone concentration in alveolar air and blood a coefficient of partition which is in agreement with that obtained by the shaking experiments with blood. This will naturally be the case only if free acetone exclusively is found in the blood. If aceto-acetic acid is also present we ought to obtain a coefficient lower than that just mentioned, on account of the fact that in the estimation in the blood a total acetone value is obtained. If, on the other hand, an active secretion of the acetone occurs, we should obtain a coefficient higher than that obtained in the shaking experiments. In the following a simple method for determining the acetone concentration in the alveolar air is described. The results of the determinations obtained by this method show that the coefficient in the case of blood/alveolar air has a value which agrees so closely with those obtained in the shaking experiments that *the elimination of the acetone may be entirely explained as a diffusion-process*. There is not the slightest reason to suppose that any secretion takes place.

The method seems to promise sufficient accuracy to permit of the use of the alveolar air analyses in determining the percentage of free acetone in the blood. Thus in a diabetic, by a combined blood estimation and analysis of alveolar air, we may arrive at an understanding of the relationship between the free acetone and the total acetone in the blood. The method has this great advantage, that the relationship between the acetone and the aceto-acetic acid can in no way be disturbed by the analysis: the separation of the free acetone from the aceto-acetic acid is effected so to speak with the organism itself as distillation apparatus.

Method.

A necessary presupposition, if these measurements are to give exact and reliable results, is that the acetone must not come into contact with rubber tubes and greased glass taps. This condition is fulfilled by the following method, which, with all its simplicity, seems to give quite satisfactory results.

The apparatus is composed of the following parts:

(*a*) A Haldane tube, made entirely of glass, length about 1·3 metres, diameter 23 mm. The mouth of the tube is produced by squeezing together one end of it, so that the tube may conveniently be stopped up by the tip of the tongue. 1 dm. inside the mouth of the tube another tube 5 cm. long is melted on at right angles to it. The diameter of this tube is 7 mm.

(*b*) A well calibrated collecting pipette for the air, about 100 cc. in capacity. This can be made out of an ordinary 100 cc. pipette by cutting off the lower end 4 cm. under the bulb and drawing it out into a 5½ cm. long capillary tube. On the tube is placed a small cup intended to serve as a mercury seal in the manner shown by Fig. 2. The other end of the pipette is attached to a rubber tube and mercury receiver. Into the rubber tube a glass tap is fitted. The rubber tube can further be compressed with a clamp.

(*c*) A test-tube prepared by fusing together the upper part of a wider and the lower part of a narrower test-tube. The upper part has a length of 4½ cm. and a diameter of 18 mm., the lower part a length of 10 cm. and a diameter of about 6 mm. (cp. Fig. 3).

(*d*) A doubly bent glass tube, one end of which is somewhat widened and the other drawn out into a 10 cm. long capillary tube of even thickness. This tube can be fixed in the test-tube by means of a perforated cork so that the point of the capillary reaches to within a couple of mm. of the bottom of the test-tube. The cork has also another perforation into which a short rectangularly bent tube is fitted (Fig. 3).

(*e*) A small V-shaped capillary tube.

When the sample of alveolar air is to be taken, the pipette is placed in such a position in regard to the Haldane tube (horizontally supported by two stands) that its capillary reaches up through the T-tube to about 1 cm. above the inner orifice of the latter (see Fig. 2). The lower orifice of the T-tube will then be closed by the mercury seal of the pipette. In this position the pipette is now filled with mercury by raising the receiver up to a certain position ascertained by experiment beforehand.

The subject of the experiment is now allowed to take the mouth of the tube between his lips. After one inspiration the nose is closed and he must now hold his breath for 20 seconds, after which he should breathe out as much air as possible into the tube. Immediately after this expiration he closes the opening of the tube with the tip of his tongue. He must now in this position breathe through the nose while the mercury is made to run out quickly from

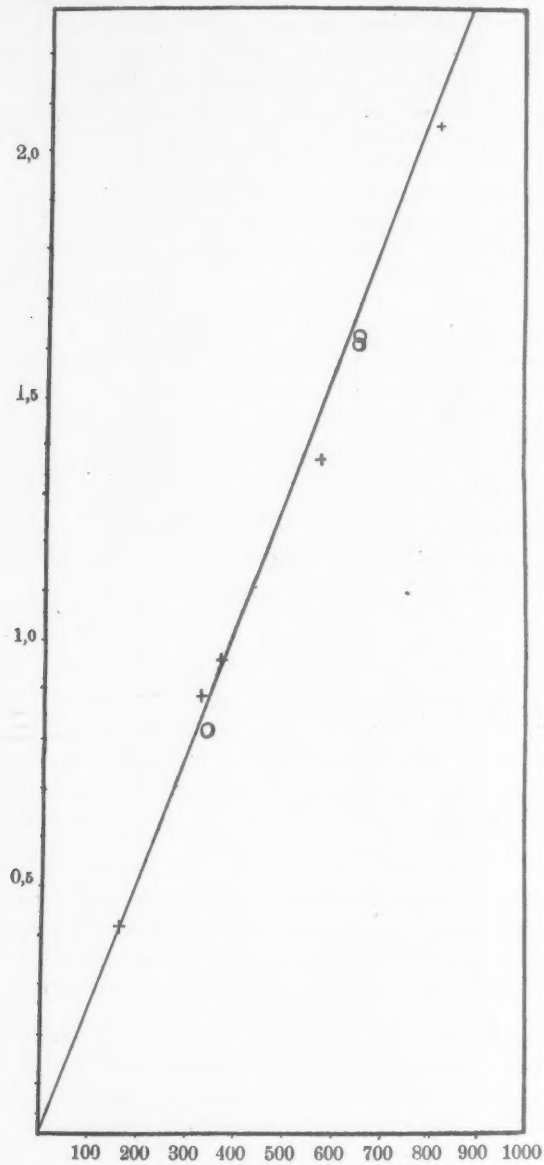

Fig. 1.

the pipette by lowering the vessel. The mercury must sink down to a certain mark on the lower tube of the pipette.

When this is done the glass tap is closed and the pipette promptly lowered so that the bent tube (*d*) can be pushed over the capillary tube of the pipette and closed by the mercury. The test-tube, containing 2 cc. of *N*/200 iodine solution and 3 cc. of *N*/1 NaOH, can be placed in position beforehand. During the whole operation the pipette should not be touched with the hands but should be held by a stand or clamps in the lowering and raising processes.

Fig. 2.

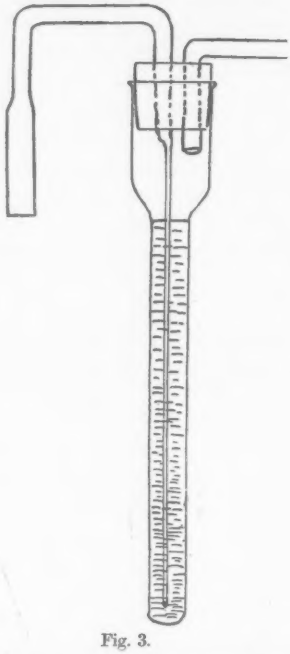

Fig. 3.

The mercury vessel is now raised, the clamp screwed on, and the glass tap opened, after which the speed of flow of the mercury is regulated by a screw-clamp so that the air is driven bubble by bubble through the alkaline iodine solution. The passage of 100 cc. ought to take about an hour. After all the air has been driven out of the pipette it is necessary to pass through the iodine solution the volume of air—amounting to about 1·5 cc.—present in the connecting tube. For this purpose the small bent glass tube in the cork of the test-tube is attached to a short piece of rubber tubing provided with a mouth-piece consisting of a capillary tube 4 cm. long. By gently sucking through this the mercury is first made to rise half a centimetre from the seal up into the connecting tube. Then the small V-shaped capillary tube is dipped into the mercury and manipulated so that one end of it emerges above the

surface into the connecting tube. A few more cc. of air are now sucked through the iodine solution, and in this way the acetone gas present in the connecting tube is washed out.

The absorption of the acetone in the alkaline iodine solution becomes in this fashion practically complete; I have not been able to find, by coupling several test-tubes together, that any acetone passes the first test-tube. The conditions for the absorption are more favourable with this apparatus than with that previously described, partly because the narrower test-tube makes the layer of fluid through which the air bubbles have to pass deeper, and partly because the stronger hydroxide increases the speed of reaction in the iodoform formation.

It was found, however, that the passage of 100 cc. of room air is sufficient to change the titre of the iodine. This change is very constant and amounts to a diminution of 0·05 cc. of *N*/200 iodine. It should always be determined by means of a blind test. It is further of great importance to provide carefully that both test-tube and connecting tube shall be extremely well cleaned and rinsed out with freshly distilled water immediately before the beginning of the experiment.

Before the titration, which in other respects proceeds in the manner previously described, the fluid is acidified with 3·5 cc. of *N*/sulphuric acid. The mixture is at once stirred and titration carried out immediately.

In the case of healthy persons, in whom an estimation by the micro-method reveals no trace of acetone in the blood, we do not obtain by the alveolar air test any greater binding of iodine than we find in the blind tests with room air. The percentage of carbonic acid in the alveolar air therefore plays no part in the estimations, and the normally occurring amount of acetone is too inconsiderable to be demonstrated.

The measurement of the acetone contained in the alveolar air is performed at room temperature. In the calculation of the acetone concentration in the air we must therefore reckon the volume of the air from room temperature to 38°, at which temperature the air is assumed to be saturated with water. This calculation is most easily performed by the aid of the tables in "Landolt and Börnstein." The air is assumed to have stood the whole time under atmospheric pressure; the slight increase of pressure arising in the lungs before the expiration into the Haldane tube may be neglected in the calculation. We add complete details for one case.

The acetone in the alveolar air after the administration of acetone.

Experiment 5. March 16, 1917. Bar. 768 mm. Room temperature 16°. Volume of the analysing pipette 95·86 cc. Subject: the author. At 10.45 a.m. about 9·5 g. of acetone were taken in 500 cc. of water. Alveolar tests at 2.22 p.m. with 20 seconds' breathing pause. The volume of the air measured, saturated with water at a pressure of 768 mm. and a temperature of 38°, was 108·3 cc.

Total quantity of acetone found: 46·9 γ.

Acetone concentration in the alveolar air: $\frac{46{\cdot}9}{108{\cdot}3} = 0{\cdot}433$ γ/cc.

Acetone concentration in the blood: at 2.20 p.m. 0·127
" " " at 2.25 p.m. 0·124 } 0·126 ‰.

$$\lambda = \frac{0{\cdot}433}{126} = 0{\cdot}00344.$$

In the same way a number of other determinations of the partition of the acetone between blood and alveolar air in experimental acetonaemia were carried out. These measurements are collected in the following table.

Table III.

Blood acetone γ/cc.	Air acetone γ/cc.	λ
222	0·59	0·00266
220	0·72	0·00327
210	0·63	0·00300
208	0·62	0·00298
199	0·59	0·00296
143	0·45	0·00315
126	0·43	0·00341
94	0·28	0·00298
63	0·16	0·00254
60	0·18	0·00300
		Average: 0·002995
		$E = \pm 0{\cdot}000257$
		$e = \pm 0{\cdot}000081$

All the determinations were carried out with the author as subject. Most of the values for the blood acetone concentration were obtained by twofold estimations. The error in these blood estimations may be estimated at about $\pm$ 15 γ per cc. of blood. The mean error for γ in the separate estimation is 0·000257. The average for λ is 0·002995 with $e = \pm$ 0·000081. The acetone percentage of the alveolar air therefore agrees very well with the concentration obtained by shaking air with blood containing acetone. In the experiments referred to (p. 383) the two values 0·00309 and 0·00329 were obtained.

The error in the estimation of λ arises partly from the error in the determination of the concentration in the blood and partly from the error in the determination of the concentration in the air. If the mean error in the blood estimation is assumed to be $\pm$ 15 γ, the error in the blood analysis alone will amount to about $\pm$ 0·00015. The air analysis cannot therefore give any appreciable error.

Hence it follows that the estimation of the free acetone in the blood can be performed with about the same accuracy by an analysis of the alveolar air as the total acetone estimation in a blood sample by means of the micro-method.

It is probable that the establishment of diffusion equilibrium between blood and alveolar air takes place almost instantaneously. In an experiment upon myself with 20 seconds' breathing pause I obtained $\lambda = 0{\cdot}00296$, and upon expiration into the Haldane tube in immediate succession to a normal expiration I found the values 0·00284 and 0·00273. With another person, B. J., as subject, the value 0·0034 was obtained after 20 seconds' breathing pause, 0·0032 after 10 seconds' and 0·0032 after a normal expiration. Since the mean error of the separate estimation is $\pm$ 0·00026 we cannot therefore demonstrate that the diffusion equilibrium has not been able to establish itself in normal respiration. Probably the difference between the acetone partial pressure of the blood and the acetone partial pressure of the alveolar air is quite inconsiderable. The experiments show at all events that a breathing pause of 20 seconds is quite sufficient to allow diffusion equilibrium to be established. This time is further taken with so wide a margin that it must be presumed that even in persons whose lungs may offer less favourable conditions for diffusion than the normal, equilibrium must be established. For further information upon these points we refer to Marie Krogh [1914].

If the acetone within the organism is found in diffusion equilibrium in the different fluids of the tissues, *the acetone in these fluids should have a partial pressure agreeing with that of the blood*. These fluids should therefore give off to the air an amount of acetone per cc. equal to that found in the alveolar air. This may be shown indirectly by the following experiment:

Experiment 6. March 16, 1917. Subject: the author. After taking a certain quantity of acetone the alveolar air contained at 2.22 p.m. 0·433 γ/cc.

Of the amount of urine collected in the bladder from 2.15–2.30 p.m. 3 cc. were shaken in a flask according to method *A*.

Concentration in the urine before shaking was 0·168 $^{0}/_{00}$ (three estimations).

Concentration in the urine after shaking was 0·085 $^{0}/_{00}$ (three estimations).

From these values λ for the urine is found to be 0·00241. The 0·168 $^{0}/_{00}$ urine can therefore give off: $168 \times 0{\cdot}00241 = 0{\cdot}405\,\gamma$ per cc. of air, which is only 7 % lower than the value found in the alveolar air. The small difference may possibly be due to the circumstance that method *A* regularly gives somewhat lower values than method *B*. The urine sample was obtained during strong diuresis (7·33), and if the value 0·00262—which was the mean value of the determinations with aqueous solutions according to method *B*—be used for λ, we obtain the value 0·437 for the acetone concentration in the air.

The acetone in the alveolar air of diabetics.

These air analyses, as has already been pointed out, render possible *the determination of the concentration of the free acetone in the blood of diabetics*.

This determination is carried out precisely in the manner previously described for the alveolar air analyses. The percentage of free acetone in the

blood is calculated by dividing the air acetone concentration by the value for λ, *i.e.* approximately 0·003[1].

The estimation of the percentage of aceto-acetic acid in the blood can be carried out in the usual way by a simultaneous blood estimation; the value of the free acetone is subtracted from the total acetone value.

In the following I give a couple of examples of these estimations of the percentage of free acetone and aceto-acetic acid in the blood of diabetics[2]. The estimations are only intended to demonstrate the possibility of the process here described. A systematic investigation of the relationship between the acetone and the aceto-acetic acid in the blood is reserved for a later work.

Experiment 7. March 14, 1917. Subject: J. N., diabetic with moderate acidosis. Breathing pause 20 seconds. Three estimations were made:

(1) At 1.30 p.m. the alveolar air contained 0·108 γ/cc.
(2) „ 2.15 „ „ „ 0·117 „
(3) „ 3.17 „ „ „ 0·135 „

From these values the free acetone in the blood was calculated, using the factor 0·003:

(1) 0·032 ‰.
(2) 0·035 ‰.
(3) 0·041 ‰.

In the same case estimations of the total acetone concentration in the blood were carried out.

2.25 p.m. $\left.\begin{matrix}0{\cdot}102\\0{\cdot}109\end{matrix}\right\}$ 0·106 ‰.

3.20 p.m. $\left.\begin{matrix}0{\cdot}121\\0{\cdot}126\end{matrix}\right\}$ 0·124 ‰.

The complete analysis of the relationship between the acetone and the aceto-acetic acid in the blood is given in the following table:

Table IV. Relationship between acetone and aceto-acetic acid in J. N. according to Exp. 6.

Time	Total acetone in blood ‰	Acetone in air γ/cc.	Free acetone in blood ‰	Combined acetone in blood ‰	Free acetone in per cent. of total acetone
1.30 p.m.	—	0·108	0·032	—	—
2.15 p.m.	0·106	0·117	0·035	0·071	33
3.17 p.m.	0·124	0·135	0·041	0·083	33

[1] A certain irregularity in the values may of course arise if the proportion between the volume of the blood corpuscles and that of the plasma departs excessively from the normal. This is however not usually the case with diabetics.

[2] In both examples the total acetone concentration in the blood is rather low, so that the estimations lie very near the limits of error of the method. Unfortunately while these experiments were being carried out I had not access to any case with a higher degree of acidosis.

Experiment 8. March 21, 1917. Subject: J. N., diabetic with moderate acidosis. Breathing pause 20 seconds. Two estimations were carried out:

Time: 2.40 p.m. 0·119 γ/cc.
3.35 p.m. 0·119 γ/cc.

The blood estimations gave the following results:

$$\text{Time: } 3.00 \left\{ \begin{matrix} 0{\cdot}073 \\ 0{\cdot}068 \end{matrix} \right\} 0{\cdot}071\ ‰.$$

$$3.40 \left\{ \begin{matrix} 0{\cdot}077 \\ 0{\cdot}080 \end{matrix} \right\} 0{\cdot}079\ ‰.$$

All the estimations are shown in Table V.

Table V. Relationship between acetone and aceto-acetic acid in J. N. according to Exp. 8.

Time	Total acetone in blood ‰	Acetone in air γ/cc.	Free acetone in blood ‰	Combined acetone in blood ‰	Free acetone in per cent. of total acetone
3.00 p.m.	0·071	0·119	0·036	0·035	51
3.40 p.m.	0·079	0·119	0·036	0·043	46

These estimations give values which may be quite well compared with those obtained by Marriott [1913]. In the four estimations published by him the pre-formed acetone constituted 63, 30, 61 and 28 % of the total acetone.

Formula for the elimination of acetone through the lungs.

A simple expression similar to that derived in Part II for the elimination of the acetone through the kidneys may also be obtained for its elimination through the lungs.

Assuming that the acetone in the alveolar air is in diffusion equilibrium with the acetone in the blood, the quantity of acetone, M, eliminated through the lungs per unit of time is evidently directly proportional to the concentration of the free acetone in the plasma and to the alveolar ventilation, *i.e.* the alveolar air exchanged during the unit of time:

$$M_l = a \,.\, V \,.\, \lambda,$$

where a = the concentration of the free acetone in the plasma;
V = the alveolar ventilation;
λ = the coefficient of partition between air and plasma.

An increase in the ventilation, *e.g.* in coma, consequently increases the amount of acetone eliminated through the lungs. Naturally this law only applies so long as the ventilation does not exceed a degree which would make it impossible for diffusion equilibrium to be set up. In the latter case the amount eliminated through the lungs is estimated not only from the factors here mentioned but also from the lung diffusion constant for acetone, and from other factors which we shall not go into here (see Marie Krogh [1914]).

The formula given above naturally applies to the free acetone only. The aceto-acetic acid does not take part in the elimination through the lungs, since its salts are not volatile. Nor is there any reason to suppose that a greater decomposition of the aceto-acetic acid takes place in the lungs than in other organs of the body[1].

As far as the mutual *relationship between the amount of acetone given off through the lungs and through the kidneys* is concerned, it is obvious that this can be estimated from the factors here mentioned.

If in the blood free acetone alone is found, the relationship will be as follows:

$$\frac{M_l}{M_n} = \frac{aV\lambda}{avk} = \frac{V\lambda}{vk}$$

where λ and κ may be regarded as constants. Besides these we therefore determine the relationship between two factors that are independent of one another: the ventilation and the quantity of urine. With increased ventilation the ratio is altered to the advantage of the elimination through the lungs; with an increased quantity of urine it is changed in the opposite direction. The quotient is always greater than 1.

If aceto-acetic acid occurs in the blood we obtain the following expression:

$$\frac{M_l}{M_t} = \frac{a.V.\lambda}{a.v.\kappa + c},$$

where a still represents the concentration of the free acetone in the blood.

Since the aceto-acetic acid (c) is given off exclusively through the kidneys, the quotient will of course become smaller the more aceto-acetic acid there is in the blood. This explains why the quotient in diabetes is less than in the experimental acetonaemia induced by the taking of acetone. In severe cases of diabetes it is small for the same reason.

Summary.

By determining the acetone concentration in the alveolar air it is established:

(1) that the elimination through the lungs is a pure diffusion process;

(2) that from a simple determination of the acetone concentration in the alveolar air it is possible to calculate the free acetone concentration in the blood.

References.

Bunsen (1877). *Gasometrische Methoden*, 2nd ed., Braunschweig.
Carveth (1899). *J. Physical Chem.*, **3**, 193.
Krogh, M. (1914). *Luftdiffusionen gennem Menneskets Lunger*, Diss., Copenhagen.
Marriott (1913). *J. Biol. Chem.*, **18**, 507.
Widmark (1917). *Acetonkoncentrationen i blod, urin och alveolärluft samt några därmed sammanhängande problem*, Gleerupska bokhandeln, Sweden.
—— (1919). *Acta Medica Scandinavica*, **52**, 87.

[1] For further details see Widmark [1919].

XXXIII. THE METABOLISM OF CARBOHYDRATES. PART I.

STEREOCHEMICAL CHANGES UNDERGONE BY EQUILIBRATED SOLUTIONS OF REDUCING SUGARS IN THE ALIMENTARY CANAL AND IN THE PERITONEAL CAVITY.

By JAMES ARTHUR HEWITT and JOHN PRYDE.

From the Physiology Laboratory, The University, St Andrews.

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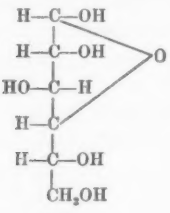
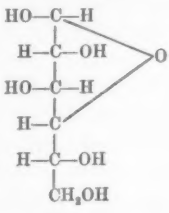
The following communication describes a number of abnormal and unexpected results encountered in an experimental study of the rates of absorption of simple carbohydrates by living tissues. As originally formulated, the scheme of research included the determination of the relative rates of absorption of simple reducing sugars such as glucose, and also of closely related derivatives such as the synthetic alkyl glucosides. The opportunity was open to use in these experiments a number of carbohydrates of exceptional purity, and it was also hoped that the influence of configuration on the rate of absorption would be determined by the employment of definite α- and β-derivatives of glucose and by the substitution of galactose and mannose for glucose in such derivatives.

At the same time it was recognised that all problems connected with the reactions of sugars are much affected by the recent recognition of the specially reactive type of which the so-called "γ-glucose" is a representative, and as the study of "γ" sugars is comparatively recent, it may be advisable to review briefly the essential facts regarding their structure.

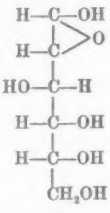
The expressions α and β are normally applied to the crystalline varieties of glucose which display downward and upward mutarotation respectively, and the following structural formulae (1) and (2) which represent the compounds as butylene oxides are applied to them:



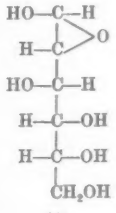
(1)



(2)



(3)



(4)

Formulae corresponding with (1) and (2) represent the isomeric crystalline methyl glucosides, but in 1914 Fischer [1914] described a third methyl glucoside which Irvine, Fyffe and Hogg [1915], who also encountered the compound simultaneously, were able to characterise as a mixture of two methyl glucosides wherein the ring is present as in an ethylene oxide.

Following Fischer's nomenclature the parent sugar was thus provisionally described as "γ-glucose" and evidence is available to show that this variety of the sugar also exists in two isomeric forms (3) and (4) which are presumably mutually related as the α- and β-glucoses.

This capacity of reducing sugars to form numerous isomerides according to the position assumed by the internal oxygen linkage is far from uncommon and what is said above seems to be of general application.

The most outstanding feature of this newly-described type of sugar is the speed and range of the reactions it undergoes. For example condensation with an alcohol to form the corresponding glucoside takes place in a few minutes and the similar reaction with acetone is practically instantaneous. By comparison, the normal forms of reducing sugars are relatively stable, inert substances and the possibility thus suggests itself that in the rapid transformations of carbohydrate in living tissues γ-sugars may play an important, if not an essential, part in both animal and vegetable metabolic processes.

In commencing the present work this possibility was not overlooked and consequently in the experiments afterwards described the amount of sugar introduced into and returned from the intestine, peritoneal cavity, etc. was estimated not only volumetrically but also polarimetrically. In this way it was possible both to determine the amount of sugar present in any solution and also to ascertain the stereochemical condition of the sugar.

It may at once be said that the original scheme of the research was realised in so far that accurate comparative measurements have been made of the rates of absorption of glucose and fructose, but this section of the work has been suspended in view of the unexpected results which indicate, with some precision, that, in contact with certain living tissues ordinary glucose (consisting of the α and β forms in equilibrium) is converted into the "γ" form in amount sufficient to be detected polarimetrically.

Incidentally it may be pointed out that quantitative estimation of glucose by reduction of cupric or other metal salts affords no indication of the presence in solution of γ-glucose. To detect this sugar use must be made either of the polarimeter or of qualitative tests involving the decolorisation of dilute solutions of $KMnO_4$ or of methylene blue [Armstrong and Hilditch, 1919]. These two colour tests have certain disadvantages when employed in experiments of the nature carried out in this work, as it is found that a dilute salt solution isotonic with mammalian tissues acquires after contact with these tissues the capacity to decolorise dilute $KMnO_4$ solutions, owing presumably to traces of organic matter. The reduction of methylene blue by living tissues is well known.

As experimental animals rabbits mostly have been used; the general results have been confirmed on rats.

The routine methods of investigation employed were:

(*a*) *Intestine.* The animal being fully anaesthetised[1], a convenient loop of intestine was isolated, ligatured where desired and two glass cannulae inserted. The loop was then thoroughly washed with Ringer-Tyrode's solution [Bayliss, 1915] made up without glucose, until the washings were clear. To facilitate this process it was found advisable to starve the animal for 24 hours before the experiment. The sugar solution was then run in and retained in the intestine for the length of time desired, usually five minutes. As a rule the abdomen was then temporarily closed by clips or forceps. The blood and nerve supply to the loop were kept intact and all solutions used were at body temperature.

In view of the extreme fragility of the intestine of the rabbit it is essential that it be handled as little as possible, and further that it be not abnormally distended.

When the specified time had elapsed the loop was emptied and washed out with salt solution two or three times; the united solutions were then filtered, made up to known volume with distilled water and examined polarimetrically as soon as possible.

The following parts of the alimentary canal have been investigated: mouth (human), duodenum, small intestine (all parts) and colon.

(*b*) *Peritoneal cavity.* In this case the sugar solutions were introduced by means of a trochar cannula and were allowed to remain in for varying periods up to 30 minutes. As it is impossible to recover all the solution run in, an amount sufficient only for polarimetric observations was withdrawn and filtered. The sugar solutions were examined as recovered and were not diluted.

Experiments have been carried out with *d*-glucose, *d*-fructose and *d*-galactose. In view however of difficulties experienced with the two latter sugars the large majority of the observations have been made with glucose. In all cases the solutions of the sugars employed were allowed to remain at the temperature of the room until the observed rotation attained a constant value. In each case this corresponded with the permanent specific rotations accepted as standard in the literature. An indication of the state of purity of the materials used may be obtained by referring to the figures given below in the experimental part for the initial rotations of the glucose and fructose in aqueous solution.

Examination of the glucose solutions after contact for five minutes with the mucous membrane of the living intestine showed that disturbance of equilibrium had taken place; initial readings corresponding with a specific

[1] All the animal experiments were carried out by one of us (J. A. H.). The remainder of the work was in greater part done by the other. At the conclusion of each animal experiment the rabbit or rat was destroyed by an overdose of anaesthetic.

rotation much lower than + 52·5° were obtained and the solutions then underwent upward mutarotation to a value corresponding with that of *d*-glucose in equilibrium.

As the original solution contained the α- and β-glucoses in equilibrium and as the former is highly dextrorotatory and the latter feebly so, the most obvious explanation is that the α form had been preferentially absorbed thus leaving excess of the β-isomeride in solution.

The range of mutarotation of pure β-glucose is from + 19° to + 52·5°, and the true initial value cannot be observed by direct experiment but is obtained by extrapolation. It follows then that even were all the α form completely absorbed and the β form unabsorbed, an initial reading corresponding with a specific rotation of + 19° could not be obtained owing to mutarotation commencing immediately the solution was withdrawn from contact with the absorbing tissue. As a certain length of time must necessarily elapse before observations can be made and as solutions on removal from intestine or peritoneal cavity cannot be used for polarimeter work without being filtered, attention was directed to minimising the time taken for this process in order to observe the mutarotation from as early a stage as possible. Even in the first experiments where no particular attention was paid to rapidity of work, extrapolation of the curves in which the specific rotations were plotted against time, indicated that mutarotation commenced from an initial specific rotation less than + 19°. Considerable difficulty was experienced in filtering the solutions rapidly owing presumably to traces of colloidal substances secreted during the experiments.

When the unavoidable delay in observing the optical changes had been reduced as far as possible, readings were obtained on the average seven minutes after removal from the intestine. On one occasion the first reading was taken in four minutes. Under these conditions extrapolation of the curves obtained indicated that mutarotation commenced at a value which was distinctly laevo. (In all cases zero time was taken as the mean of the times of commencing and finishing removal of the sugar solution from contact with the living tissue.) In the great majority of cases also, the first reading obtained corresponded with a specific rotation much too close to + 19° to be due to the presence of β-glucose alone. Not only so, but on one occasion in which glucose was used a laevo initial value was recorded, and on several occasions the initial specific rotations actually obtained lay between 0° and + 19°.

It is clear therefore that the upward mutarotation observed on removal from the intestine cannot solely be due to a preferential absorption of α-glucose with the subsequent establishment of equilibrium *in vitro*.

One deduction from the experimental results described is that, in contact with certain living tissues, glucose solutions must undergo a downward mutarotation, and if the above explanation be accepted, this change is due in part at least to the transient formation of γ-glucose. This is supported by the additional observation that *N*/100 $KMnO_4$ in neutral solution is de-

colorised appreciably more rapidly by glucose after contact with the living intestine, than by either glucose of the same concentration in aqueous solution, *or by washings from the intestine*, and further that the absorption of glucose takes place in the peritoneal cavity without any stereochemical changes being manifest.

A point of considerable importance is that the sugar solutions on withdrawal from the intestine are of necessity alkaline, whilst on the other hand it would appear that faintly acid conditions are most favourable to the formation of γ-sugars. The possibility cannot be excluded however, that this particular type may be formed in feebly alkaline media so long as the alkalinity is lower than that found necessary by Lobry de Bruyn to promote the aldose $\rightleftarrows$ ketose transformation.

During the course of each experiment the sugar solutions employed were inevitably contaminated both with intestinal secretions and also with Ringer-Tyrode's solution. It became a matter of importance therefore to determine the effect, if any, of these substances on a glucose solution in equilibrium and on the rate of mutarotation of a freshly prepared solution of glucose. Further as the experiments were carried out at body-temperature (37°), and as the rotations were taken at 11°–15°, the effect of this alteration in temperature must be considered.

It has not been found possible to effect an alteration in specific rotation of a glucose solution in equilibrium either by the addition of Ringer-Tyrode's solution, of extracts of intestinal mucous membrane or by Ringer-Tyrode washings from the intestine. While the final equilibrium of a glucose solution is not disturbed by these substances the speed of mutarotation of a freshly prepared solution of *d*-glucose is very appreciably increased. The final permanent value obtained is the same as that of α-glucose dissolved in distilled water.

Temperature variations of from 22°–26° may, in the case of glucose, be ignored [v. Lippmann, 1904]. Even in the case of fructose, which is notoriously sensitive in this respect, only at 87·3° does the specific rotation reach the value of −52·5° [Armstrong, 1919], and it is considered that the changes in optical activity obtained with this sugar cannot wholly be due to temperature variations. Pending further work with a constant temperature apparatus, it is not proposed to discuss either fructose or galactose in greater detail.

The fact that the final permanent specific rotation attained after removal from the intestine is the same as that given by the sugar before being introduced precludes also any possibility of these alterations in rotatory power being due to formation of disaccharides or other sugar complexes. As an additional check the concentration of glucose as estimated from the optical value was verified volumetrically by Benedict's method. Close agreements were found in each case.

It should be mentioned that in some of the experiments in which glucose solutions were employed the mutarotation effects were ill-defined and in

some cases were completely negative; it is thus advisable to describe as closely as possible the conditions adopted when positive results were obtained.

In experiments on the absorption of glucose by the small intestine, solutions have been employed hyper-, iso- and hypo-tonic to mammalian tissues. In hypo-tonic solutions only have the changes been as described. In solutions of higher concentration the range of mutarotation *in vitro* has not been so great. In the large intestine also, with dilute solutions of glucose the results obtained were in no way so well-marked as in the case of glucose of similar concentration in the small intestine.

Examination of glucose solutions retained intra-peritoneally for 15–30 minutes showed that the equilibrium of the various isomerides was not disturbed.

After being retained in the mouth (human) for five minutes polarimetric evidence was obtained in the case of glucose that stereoisomeric changes had taken place. Mutarotation was then observed similar to, but much less well-marked than, that noted with dilute solutions of glucose in the small intestine.

Experimental.

The general methods adopted in these experiments have been described above.

In connection with the difficulty experienced at times in obtaining solutions sufficiently clear for polarimetric observations it may be said that after many trials of various types of filter papers, shaking with silica and centrifuging, etc. the most satisfactory procedure was found in filtering directly into a graduated flask, under very slight suction, through a "filter-hat" manufactured by Messrs W. and R. Balston, Ltd. These filter-hats are thick and spongy in texture and, provided they are well washed with water before use, yield, in the great majority of cases, solutions clear enough for accurate readings in a 2 dcm. tube with sodium light.

In certain very few cases, even after careful filtration, the solution was not sufficiently clear for sodium light to be used and recourse had to be made either to incandescent gas flame or to the electric arc. As a routine proceeding all three sources of light were available by rotating the polarimeter on the table to the light desired. Monochromatic sodium light was always tried first and then in order the incandescent flame and the arc. In the latter case light filters for yellow light were employed [Landolt, 1898].

Polarimetric examination of the glucose and fructose employed in these experiments gave the following initial specific rotations three minutes after commencement of solution of the sugar:

$$d\text{-glucose},\ 2{\cdot}868\ \%\ [\alpha]_D^{12^\circ} = +\ 103{\cdot}4^\circ$$

$$d\text{-fructose},\ 3{\cdot}215\ \%\ [\alpha]_D^{12^\circ} = -\ 107{\cdot}8^\circ.$$

At the end of $1\frac{1}{2}$ hours the fructose had not attained equilibrium, and after six hours the glucose gave readings corresponding with a specific rotation

of + 54·9°. This is an important point, as the speed of the optical change varies enormously according to the nature of the glass vessels employed and to other unavoidable factors.

On the graph given below (Fig. 1), are plotted:

I. The velocity of mutarotation of *d*-glucose in aqueous solution.
II. „ „ „ filtered intestinal washings.
III. „ „ „ Ringer-Tyrode's solution.

It will be seen that the equilibrium rotation is attained more rapidly in the presence of either of these two solutions than when the glucose is dissolved in water only.

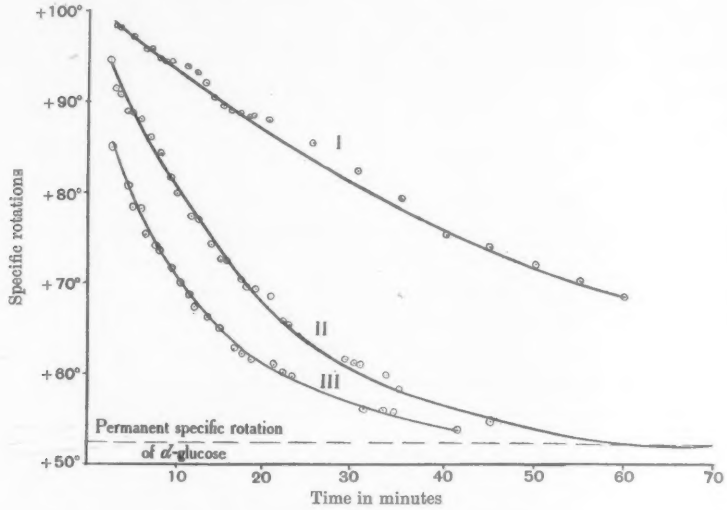


Fig. 1.

I. Glucose 2·907 % in aqueous solution.
II. „ 2·924 % in filtered intestinal washings.
III. „ 2·887 % in Ringer-Tyrode's solution.

1. *Small intestine.*

(*a*) *d*-Glucose, hypo-tonic aqueous solutions; 1·571 % and 2·120 %.

Graphic records of three experiments which show the optical changes most markedly are given below (Figs. 2, 3 and 4). On these are plotted the specific rotations at varying intervals after withdrawal of the solution from the intestine. After the readings had become steady a trace of caustic soda was added and the optical value then observed was taken as the permanent rotation.

As stated above, the final readings before and after the addition of the trace of alkali gave a concentration of sugar which agreed remarkably well with the concentration as determined from volumetric estimations. The

Benedict's solution employed was previously standardised against the sugars used.

(*b*) *d*-Glucose, iso- and hyper-tonic aqueous solution, 5·258 % and 8·040 %.

Regarding these it can be said that evidences of disturbance of equilibrium are shown, but are slight. In no case has a reading indicating a specific rotation below + 40° been obtained under similar conditions to those already described above.

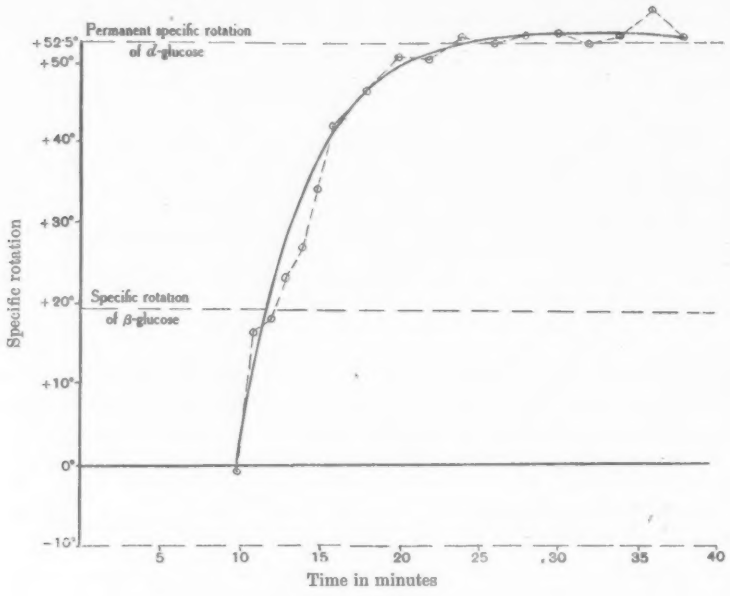


Fig. 2. *d*-Glucose.

In this and in all subsequent graphs (except no. 5) the values plotted represent the average of at least two readings taken in as short a time as possible. Readings were continuous on the part of one author, verified as a rule at intervals by the other. Below each graph are given the rotations from which the specific rotations were calculated. All rotations are dextro unless otherwise stated.

Observed rotations

−0·017°, 0·156°, 0·166°, 0·213°, 0·247°, 0·315°, 0·383°, 0·425°, 0·468°, 0·468°, 0·500°, 0·485°, 0·500°, 0·500°, 0·485°, 0·490°, 0·520°, 0·500°.

After addition of a trace of alkali. Mean of nine readings, 0·483°.

(*c*) *d*-Fructose, hypo-tonic aqueous solution, 2·01 %.

One graph is reproduced (Fig. 5). Other experiments have shown, in the initial observations, greater deviation from the equilibrium rotation, but the tracings are complicated by what appears to be the existence of two simultaneous reactions.

(*d*) *d*-Galactose, hypo-tonic aqueous solution, 2·317 %.

Evidence has been obtained that the specific rotation of this sugar in equilibrium is disturbed through contact with living intestine. The alteration in rotation observed is, however, small.

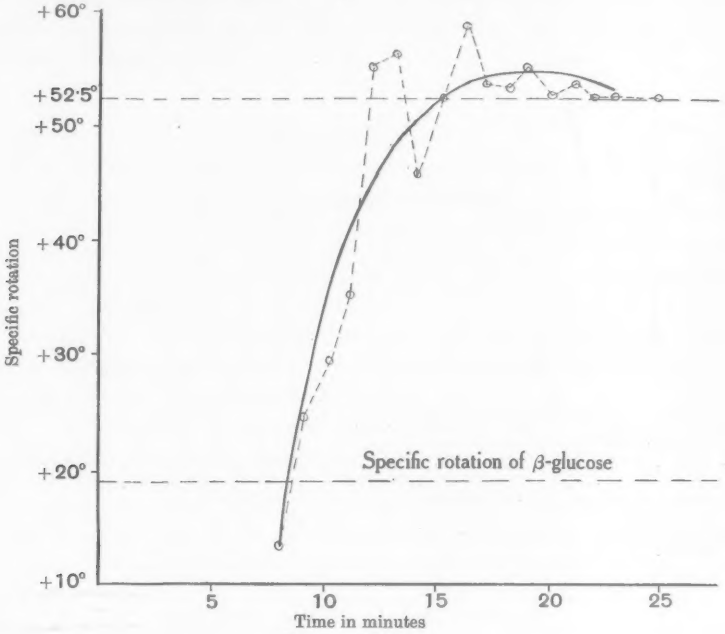


Fig. 3. *d*-Glucose.

Observed rotations

0·083°, 0·150°, 0·250°, 0·217°, 0·342°, 0·350°, 0·283°, 0·325°, 0·367°, 0·333°, 0·333°, 0·342°, 0·325°, 0·333°, 0·325°, 0·325°.

After addition of a trace of alkali. Mean of seven readings, 0·325°.

2. *Large intestine.*

In the rabbit with a 3 % solution of glucose, an initial specific rotation of + 43·03° has been obtained nine minutes after removal from the intestine. After 14 minutes the value + 52·31° was found. In experiments where the normal activity of the intestine was impaired, either by killing the animal previously or by interrupting the blood supply to the observed loop, no alteration of rotation was detected.

3. *Mouth* (*human*).

Dilute glucose solutions on being examined after being retained in the mouth for five minutes show upward mutarotation. The range of the mutarotation is small.

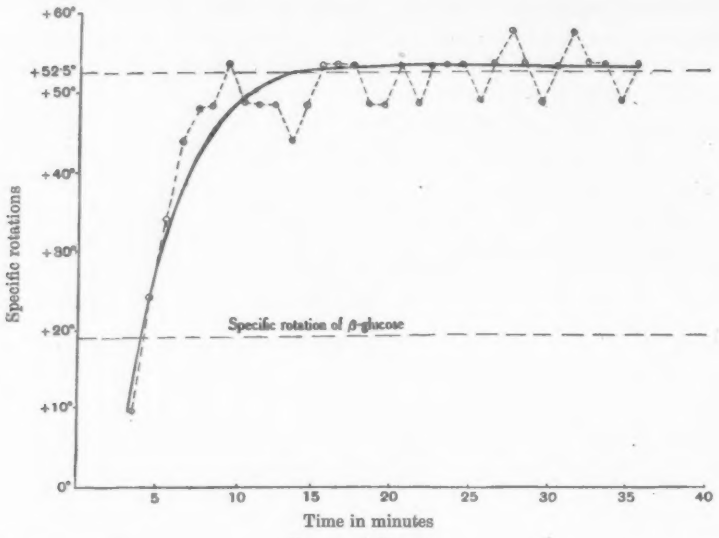


Fig. 4. *d*-Glucose.

Observed rotations

0·033°, 0·083°, 0·117°, 0·150°, 0·167°, 0·167°, 0·183°, 0·167°, 0·167°, 0·167°, 0·150°, 0·167°, 0·183°, 0·183°, 0·183°, 0·167°, 0·167°, 0·183°, 0·167°, 0·183°, 0·183°, 0·183°, 0·167°, 0·183°, 0·200°, 0·183°, 0·167°, 0·183°, 0·200°, 0·183°, 0·183°, 0·167°, 0·183°.

After addition of a trace of alkali. Mean of 10 readings, 0·180°.

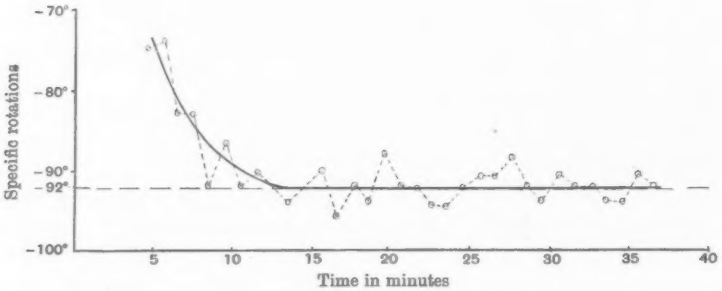


Fig. 5. *d*-Fructose.

Observed rotations

−0·070°, −0·066°, −0·750°, −0·750°, −0·833°, −0·783°, −0·833°, −0·816°, −0·833°, −0·850°, −0·833°, −0·816°, −0·866°, −0·833°, −0·850°, −0·800°, −0·833°, −0·833°, −0·850°, −0·850°, −0·833°, −0·816°, −0·816°, −0·800°, −0·833°, −0·850°, −0·816°, −0·833°, −0·833°, −0·850°, −0·850°, −0·816°, −0·833°.

After addition of a trace of alkali. Mean of 14 readings, −0·831°.

Considerable difficulty was experienced in filtering the solutions owing to admixture of saliva.

4. *Peritoneal cavity.*

No evidence has been obtained from polarimetric observation that alteration of rotation of glucose in equilibrium takes place in the peritoneal cavity of either the rabbit or the rat.

Summary and Conclusions.

1. Solutions of *d*-glucose in equilibrium when introduced into the intestine of the living animal undergo stereochemical changes.

2. The change in the intestine is a rapid downward mutarotation of the sugar solution to optical values corresponding with specific rotations much below + 52·5°. Several specific rotations below + 19°, and one laevo rotation have been recorded.

3. After withdrawal from the intestine these solutions undergo slower upward mutarotation to a permanent value corresponding with the specific rotation of α- and β-glucoses in equilibrium.

4. The stereochemical changes in the intestine cannot be attributed to the production in excess of β-glucose through preferential absorption of the α form, or to the formation of sugar complexes such as disaccharides.

5. Contact with the mucous membrane of the living intestine probably effects the production of γ-glucose in excess of any amount normally present in glucose solutions which have attained a permanent specific rotation.

6. The above stereochemical changes do not occur in sugar solutions which have been introduced into the peritoneal cavity.

The authors thank Professor J. C. Irvine, F.R.S. for his constant advice and criticism on the purely chemical aspects of the work, and Professor P. T. Herring for similar kindness on the physiological side.

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References.

Armstrong (1919). *The simple Carbohydrates and the Glucosides*, Longmans and Co., Ltd.
—— and Hilditch (1919). *J. Chem. Soc.*, **115**, 1410.
Bayliss (1915). *Principles of General Physiology*, Longmans and Co., Ltd.
Fischer (1914). *Ber.*, **47**, 1980.
Irvine, Fyffe and Hogg (1915). *J. Chem. Soc.*, **107**, 524.
Landolt (1898). *Das optische Drehungsvermögen.*
v. Lippmann (1904). *Die Chemie der Zuckerarten.*

XXXIV. THE EXISTENCE IN THE BILE OF AN INHIBITOR FOR HEPATIC ESTERASE, AND ITS NATURE.

By GEORGE MACFEAT WISHART.

From the Department of Physiology, University of Glasgow.

(*Received March 23rd, 1920.*)

It is generally accepted that the addition of bile to pancreatic juice accelerates its lipolytic activity. As is well known, there can be extracted from the liver an enzyme capable of rapidly hydrolysing the lower esters, and the object of the present research was to discover whether a similar effect would be produced on this esterase by the addition of bile.

The research was commenced in 1914 at the suggestion of Prof. Cathcart, and the greater part of the work was done at that time. I was then unaware of Loevenhart's [1906, 1, 2] investigations on the effect of bile-salts on the liver esterase. My work had to be left uncompleted, and it was not until its resumption at the end of the past year that Loevenhart's paper on the above subject came to my notice.

Various methods of preparing the liver extract were tried, the first attempts being directed towards obtaining a dry preparation of liver by extraction with acetone and ether, or alcohol and ether. Such dry preparations were found, however, to possess very slight esterolytic properties (Exp. 1). This is in accordance with the experiences of Loevenhart [1906, 2, p. 433].

Exp. No. 1. *Cat's Liver.*

Quantities used:		Minutes	Cc. *N*/10 NaOH
		5	0·7
Ethyl butyrate	0·25 cc.	10	1·1
Extract	0·5 g.	15	1·3
Water	5·0 cc.	20	1·5
Phenolphthalein	4 drops	25	1·6
		30	1·6

The method of obtaining a dry active preparation described by Loevenhart [1906, 2, p. 432], which is a modification of a method cited by Magnus [1904], was not tried. Ramond's [1904] ether extraction method was found to be unsatisfactory.

10 % aqueous extracts were found to be quite satisfactory for the work in view, and were generally used. 5 g. of liver were ground in a mortar with a suitable amount of sand and water, and the extract made up to 50 cc. with water. The whole was then strained through muslin into a stoppered bottle and the bottle well shaken before pipetting off any quantity for use.

Such extracts were found to be highly active, and, despite the fact that, even by thorough shaking it would seem impossible to obtain a homogeneous mixture, successive quantities of equal amount withdrawn from the bottle were found to have practically the same esterolytic activity (Exps. 2 and 3). If a small amount of toluene were added to the extract, the diminution in its activity with age was remarkably slow, as shown in Exp. 4. Throughout the work, however, fresh extracts were used for each experiment.

Exp. No. 2. *Cat's Liver.* 10 % *Extract.*

Quantities in each tube:		Minutes	Cc. *N*/10 NaOH	
			First 5 cc.	Second 5 cc.
Ethyl butyrate	0·5 cc.	5	1·4	1·4
Extract	5·0 cc.	10	2·5	2·6
Water	5·0 cc.	15	3·6	3·5
Phenolphthalein	4 drops	20	4·6	4·5
		25	5·3	5·4
		30	6·1	6·0

Exp. No. 3. *Cat's Liver.* 10 % *Extract.*

Quantities:	Minutes	Cc. *N*/10 NaOH	
		First 5 cc.	Second 5 cc.
As in No. 2	5	2·3	2·2
	10	4·3	4·2
	15	6·0	5·9
	20	7·6	7·5
	25	8·9	8·7
	30	9·9	9·8

Exp. No. 4. *Cat's Liver.* 10 % *Extract.*

Quantities:	Minutes	Cc. *N*/10 NaOH	
		14th Dec.	22nd Dec.
As in No. 2	5	1·3	1·4
	10	2·9	2·6
	15	4·5	3·8
	20	5·6	4·6
	25	6·7	5·2
	30	7·7	5·7

Ethyl butyrate was the ester used in all cases, and, as the butyric acid set free rapidly destroyed the hepatic enzyme, the following method which, to a certain extent, obviated this, and also, it was thought, gave a closer approximation to the *in vivo* conditions, was adopted.

Tubes containing the ester, indicator (phenolphthalein), and water were placed in a water-bath at 38° for five minutes. The extract was then added and the contents of the tubes exactly neutralised with *N*/10 NaOH. Having been replaced in the water-bath, a burette was fixed above each containing *N*/10 NaOH, and titrations of the acidity developed were carried out every five minutes during a period of half-an-hour. Under such conditions esterolysis was found to be much more active than when titration of the mixture was performed only at the end of the period of incubation.

A. Inhibition of the Hepatic Esterase by Bile.

The following experiments show the effect of adding bile to the enzyme-containing liver extract (Nos. 5 to 13).

A summary of all results with the conclusions drawn therefrom will be found at the end of the paper.

Exp. No. 5. *Dog's Liver.* 10 % *Extract.*

Quantities in each tube:

Ethyl butyrate	0·25 cc.
Extract	1·0 cc.
Water	4·0 cc.
Phenolphthalein	4 drops
Amount of bile used	0·15 cc.

	Cc. *N*/10 NaOH			
	Without bile		With bile	
Minutes	Lobe A	Lobe B	Lobe A	Lobe B
5	0·9	0·9	0·7	0·6
10	1·4	1·4	0·8	0·65
15	1·65	1·6	0·8	0·65
20	1·7	1·7	0·8	0·65
25	1·8	1·75	0·85	0·65
30	1·85	1·8	0·85	0·7

Exp. No. 6. *Cat's Liver.* 10 % *Extract.*

Quantities:
As in No. 5

	Cc. *N*/10 NaOH	
Minutes	Without bile	With bile
5	0·3	0·3
10	0·5	0·35
15	0·7	0·35
20	0·85	0·4
25	0·95	0·45
30	1·05	0·5

Exp. No. 7. *Same extract as No.* 6. *Tubes kept at room temperature.*

Quantities:
As in No. 5

	Cc. *N*/10 NaOH	
Minutes	Without bile	With bile
5	0·2	0·2
10	0·35	0·3
15	0·45	0·4
20	0·55	0·45
25	0·65	0·55
30	0·75	0·6

Exp. No. 8. *Rabbit's Liver.* 10 % *Extract.*

Quantities:
As in No. 5

	Cc. *N*/10 NaOH			
	Without bile		With bile	
Minutes	Lobe A	Lobe B	Lobe A	Lobe B
5	1·0	0·95	0·6	0·55
10	1·7	1·7	0·85	0·85
15	2·3	2·3	1·05	1·05
20	2·85	2·8	1·2	1·25
25	3·3	3·25	1·4	1·4
30	3·65	3·6	1·55	1·5

Exp. No. 9. *Rabbit's Liver.* 10 % *Extract.*

	Minutes	Cc. *N*/10 NaOH: Without bile	With bile
Quantities: As in No. 5	5	0·5	0·3
	10	1·0	0·45
	15	1·35	0·65
	20	1·75	0·85
	25	2·1	1·0
	30	2·45	1·15

Exp. No. 10. *Rabbit's Liver.* 10 % *Extract.*

	Minutes	Cc. *N*/10 NaOH: Without bile	With bile
Quantities: As in No. 5	5	0·7	0·65
	10	1·3	1·1
	15	1·8	1·5
	20	2·3	1·9
	25	2·7	2·2
	30	3·1	2·5

Exp. No. 11. *Rabbit's Liver.* 10 % *Extract.*

	Minutes	Cc. *N*/10 NaOH: Without bile	With bile
Quantities: As in No. 5 except that 0·5 cc. bile used	5	0·9	0·2
	10	1·75	0·2
	15	2·3	0·2
	20	2·9	0·2
	25	3·2	0·3
	30	3·6	0·3

Exp. No. 12. *Rabbit's Liver.* 10 % *Extract.*

	Minutes	Cc. *N*/10 NaOH: Without bile	With bile
Quantities: As in No. 11	5	0·9	0·2
	10	1·5	0·3
	15	1·95	0·3
	20	2·5	0·4
	25	2·9	0·4
	30	3·3	0·4

Exp. No. 13. *Guinea-pig's Liver.* 10 % *Extract.*

	Minutes	Cc. *N*/50 NaOH: Without bile	With bile
Quantities: As in No. 5 *N*/50 NaOH used for titration	5	1·8	1·2
	10	3·0	1·9
	15	3·7	2·6
	20	4·2	3·2
	25	4·5	3·5
	30	4·8	3·7

That the dilution due to the addition of the bile has a negligible effect on the reaction is shown in Exp. No. 14.

Exp. No. 14. *Cat's Liver.* 10 % *Extract.*

	Cc. *N*/10 NaOH	
Minutes	Without water	With 0·15 cc. water
Quantities:	5	1·45	1·4
As in No. 5	10	2·55	2·55
	15	3·45	3·4
	20	4·3	4·3
	25	5·05	5·0
	30	5·75	5·65

Control tubes containing ester without extract, and extract without ester, treated in an exactly similar manner, developed in half-an-hour an acidity of comparatively negligible amount.

B. Is the Inhibiting Substance Specific for each Species?

Exps. Nos. 15 to 21 were carried out in an endeavour to find whether the liver extract of one animal would be inhibited by the bile of another animal of a different species, and if the inhibition by the foreign bile would be as marked as that produced by the animal's own bile.

Exp. No. 15. *Livers from Cat, Dog, and Rabbit.* 10 % *Extracts.*

A. Cat's Liver Extract. Quantities:—As in No. 5.

	Cc. *N*/10 NaOH			
Minutes	Without bile	With cat's bile	With dog's bile	With rabbit's bile
5	0·35	0·25	0·25	0·35
10	0·55	0·35	0·35	0·5
15	0·65	0·35	0·35	0·6
20	0·8	0·35	0·4	0·7
25	0·85	0·4	0·5	0·8
30	0·9	0·45	0·5	0·8

B. Dog's Liver Extract. Quantities:—As in No. 5.

	Cc. *N*/10 NaOH			
Minutes	Without bile	With dog's bile	With cat's bile	With rabbit's bile
5	0·65	0·4	0·5	0·5
10	1·0	0·45	0·65	0·7
15	1·15	0·45	0·7	0·85
20	1·25	0·5	0·75	0·9
25	1·35	0·5	0·75	0·9
30	1·4	0·5	0·85	1·0

C. Rabbit's Liver Extract. Quantities:—As in No. 5.

	Cc. *N*/10 NaOH			
Minutes	Without bile	With rabbit's bile	With dog's bile	With cat's bile
5	1·1	0·65	0·75	0·85
10	2·2	1·05	1·3	1·6
15	3·15	1·4	1·7	2·3
20	3·9	1·65	2·1	2·8
25	4·5	1·85	2·2	3·4
30	5·1	2·0	2·3	3·9

Exp. No. 16. *Cat's Liver Extract.* Quantities:—As in No. 2. Bile 0·15 cc.

Minutes	Cc. *N*/10 NaOH			
	Without bile	With cat's bile	With rabbit's bile	With dog's bile
5	3·9	1·6	2·0	2·6
10	8·1	3·0	4·4	5·7
15	12·0	4·5	6·9	8·8
20	15·4	5·8	9·7	12·0
25	18·4	7·1	12·1	15·1
30	20·9	8·2	14·4	17·6

Exp. No. 17. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 16.

Minutes	Cc. *N*/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	1·7	1·2	1·2
10	3·2	2·2	2·3
15	4·7	3·1	3·3
20	6·0	3·5	4·0
25	7·0	3·8	4·3
30	7·9	4·1	4·8

Exp. No. 18. *Rabbit's Liver.* 20 % *Extract.* Quantities:—As in No. 16.

Minutes	Cc. *N*/10 NaOH		
	Without bile	With rabbit's bile	With ox bile
5	2·4	1·6	1·6
10	5·5	2·8	3·4
15	8·5	4·0	5·0
20	10·9	5·05	6·4
25	13·5	6·1	7·8
30	15·7	6·9	9·0

Exp. No. 19. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 16.

Minutes	Cc. *N*/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	1·0	1·1	0·75
10	1·9	2·2	1·7
15	3·0	3·2	2·7
20	4·2	4·3	3·7
25	5·3	5·0	4·6
30	5·9	5·3	5·3

Exp. No. 20. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 16.

Minutes	Cc. *N*/10 NaOH		
	Without bile	With cat's bile	With ox bile
5	2·3	1·7	1·9
10	4·3	3·7	3·8
15	6·0	5·4	5·3
20	7·6	6·9	6·3
25	8·9	8·0	7·4
30	9·9	9·0	8·4

Exp. No. 21. *Livers from Dog and Cat.* 10 % *Extracts.*

A. Dog's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. *N*/10 NaOH		
	Without bile	With dog's bile	With cat's bile
5	0·7	0·4	0·7
10	1·2	0·5	1·1
15	1·5	0·55	1·3
20	1·7	0·6	1·4
25	1·8	0·6	1·5
30	1·95	0·65	1·55

B. Cat's Liver Extract. Quantities:—As in No. 5.

Minutes	Cc. *N*/10 NaOH		
	Without bile	With cat's bile	With dog's bile
5	0·3	0·25	0·2
10	0·45	0·4	0·2
15	0·6	0·5	0·3
20	0·7	0·6	0·35
25	0·8	0·65	0·35
30	0·85	0·65	0·4

C. The Nature of the Inhibitor.

It has been shown by various workers [von Fürth and Schütz, 1906; Hewlett, 1905; Loevenhart and Souder, 1906; and others] that a solution of bile-salts will produce an acceleration of the hydrolysis of both fats and the lower esters by pancreatic juice, similar to that obtained on adding the whole bile. Further it has been demonstrated by Magnus [1906] that bile-salts synthetically prepared have the same action. The same author showed in a previous paper [1904] how the esterolytic ferment of the liver could be rendered inactive by dialysis, and that the dialysable portion was thermostable, soluble in alcohol but insoluble in ether, and destroyed by incineration. Magnus used as zymolyte amyl salicylate and his results were confirmed by Loevenhart [1906, 1, 2], who further discovered that the activity of the non-dialysable portion could be restored by the addition of bile-salts.

Loevenhart found, however, that while this was true of the hydrolysis of amyl salicylate, ethyl butyrate behaved in a different manner. Dialysis did not cause the liver extract to lose its property of hydrolysing the latter ester; and when bile-salts were added to a non-dialysed liver extract its activity towards ethyl butyrate was diminished.

The following experiments were performed without knowledge of this part of Loevenhart's work and are fully confirmatory of his results.

Exps. 22 to 24 were carried out to find if the inhibitory substance were dialysable. A quantity of bile was dialysed and the effect on the esterase of both dialysate and residue determined (Nos. 22 and 23). Part of the dialysate was again dialysed. The effect of this double dialysate is shown in No. 24.

Exp. No. 22. *Dog's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

Minutes	Cc. *N*/10 NaOH		
	Normal	Dialysate added	Residue added
5	1·5	1·4	1·2
10	2·7	2·0	1·8
15	3·2	2·6	2·1
20	3·9	3·0	2·5
25	4·2	3·3	2·8
30	4·6	3·7	3·1

Exp. No. 23. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

Minutes	Cc. *N*/10 NaOH		
	Normal	Dialysate added	Residue added
5	2·1	1·9	2·2
10	4·5	4·1	4·1
15	7·0	6·1	6·0
20	9·0	7·8	7·6
25	10·6	9·2	9·2
30	12·0	10·6	10·7

Exp. No. 24. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

Minutes	Cc. *N*/10 NaOH	
	Normal	Double dialysate added
5	2·2	2·2
10	4·5	4·4
15	6·8	6·5
20	8·7	8·2
25	10·5	9·5
30	12·0	10·8

It is evident that the inhibitory substance is dialysable, though the residue from dialysis still shows considerable inhibitory power. In the light of subsequent work it would seem that the bile was not allowed to dialyse for a sufficient length of time.

An alcoholic extract of the bile was made, the extract evaporated to dryness, and the material so obtained tested for inhibitory power. Exp. No. 25 shows the marked inhibition obtained on adding a small quantity of this dried alcoholic extract.

Exp. No. 25. *Rabbit's Liver.* 20 % *Extract.* Quantities:—As in No. 2.

Minutes	Cc. *N*/10 NaOH	
	Normal	Alcoholic extract added
5	2·4	1·4
10	5·3	2·0
15	8·5	2·8
20	11·1	3·3
25	13·5	3·8
30	15·7	4·4

Exps. Nos. 26 to 30 show the inhibition obtained on adding progressively smaller quantities of the evaporated alcoholic extract (A.E.). In No. 30 only 1 mg. of the material was used. In these only the difference between the acidity of the normal tube and the tube with alcoholic extract added (*i.e.* the amount of inhibition expressed in cc. *N*/10 NaOH) is given.

Cat's Liver. 10 % *Extract.*

	Inhibition in cc. *N*/10 NaOH				
Minutes	Exp. 26 ca. 10 mg. A.E.	Exp. 27 ca. 8 mg. A.E.	Exp. 28 ca. 6 mg. A.E.	Exp. 29 ca. 4 mg. A.E.	Exp. 30 1 mg. A.E.
5	0·3	0·4	0·3	0·0	0·0
10	0·6	0·7	0·5	0·0	0·2
15	1·0	1·0	0·6	0·1	0·2
20	1·1	1·2	0·6	0·1	0·2
25	1·4	1·2	0·8	0·4	0·2
30	1·4	1·3	0·9	0·5	0·1

That the alcohol-soluble portion of the bile contains all the inhibitory substance, none remaining in the residue, is shown in Nos. 31 and 32.

Exps. Nos. 31 *and* 32. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Cc. *N*/10 NaOH		Cc. *N*/10 NaOH	
Minutes	Normal	Residue from alcoholic extraction	Normal	Residue from alcoholic extraction
5	1·8	1·9	1·9	2·0
10	4·0	4·0	4·1	3·9
15	6·2	6·1	6·3	6·2
20	8·0	8·0	8·1	8·0
25	9·8	9·7	10·0	9·9
30	11·3	11·2	11·5	11·5

A solution in water of the dried alcoholic extract was then treated with ether. The precipitate was dried and the filtrate evaporated to dryness. Solutions in water of the material from each were investigated. The inhibitory substance was evidently insoluble in ether (No. 33).

Exp. No. 33. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Cc. *N*/10 NaOH		
Minutes	Normal	Filtrate	Residue
5	2·5	2·6	1·0
10	5·3	5·8	1·9
15	7·4	7·8	2·5
20	9·3	9·6	3·2
25	11·5	11·8	3·7
30	13·7	14·1	4·2

Exps. Nos. 34 and 35 show the inhibition obtained, using a 3 % aqueous solution of Plattner's crystals, while No. 36 shows that the inhibitory power is not diminished but rather increased when the bile-salts are purified by recrystallisation.

Exps. Nos. 34 *and* 35. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Cc. *N*/10 NaOH		Cc. *N*/10 NaOH	
Minutes	Normal	0·15 cc. bile-salt solution added	Normal	0·15 cc. bile-salt solution added
5	1·4	1·1	1·4	1·1
10	2·5	2·0	2·6	2·0
15	3·6	2·8	3·5	2·8
20	4·6	3·4	4·5	3·6
25	5·3	3·8	5·4	4·5
30	6·1	4·2	6·0	5·0

Exp. No. 36. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Cc. *N*/10 NaOH		
Minutes	Normal	Before recrystallisation	After recrystallisation
5	1·6	1·2	1·3
10	2·8	2·4	2·3
15	4·3	3·5	3·4
20	5·5	4·5	4·4
25	6·8	5·6	5·4
30	7·8	6·4	6·2

The sensitiveness of the hydrolysis of ethyl butyrate to the inhibiting action of bile-salts was found to be very considerable (Nos. 37 to 43, amounts of inhibition in cc. *N*/10 NaOH only given). In this connection Loevenhart [1906, 2] found that concentrations of 0·02 % and under, accelerated rather than inhibited the reaction. I have been unable to confirm this, finding inhibition to be produced in concentrations as low as 0·005 %. Concentrations lower than this were not tried, as it was thought that the method of estimating the activity of the enzyme was not sufficiently accurate to allow of this being done.

Exps. Nos. 37 *to* 43. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Inhibition caused by bile-salts in concentration of:						
Minutes	2 %	1 %	0·5 %	0·1 %	0·05 %	0·01 %	0·005 %
5	0·8	0·5	0·4	0·2	0·1	0·0	0·0
10	1·5	1·6	1·1	0·3	0·2	0·0	0·1
15	2·5	2·6	1·7	0·8	0·5	0·1	0·4
20	3·5	3·5	2·7	1·1	0·7	0·3	0·4
25	4·4	4·2	3·4	1·6	1·0	0·6	0·6
30	5·2	5·4	4·1	2·4	1·2	0·8	0·7

D. Other Inhibitors.

Rosenheim and Shaw-McKenzie [1910] found that sodium cholate, saponin, and serum, among other substances, acted as accelerators to the pancreatic lipase in its action on olive oil. The presence of cholesterol inhibited this acceleration. Von Fürth and Schütz [1906] also showed that sodium cholate was an active accelerator of the hydrolysis of fat by pancreatic juice. The activation of pancreatic lipase by saponin was investigated by Flohr [1919],

who showed that the acceleration produced was proportional to the lowering of surface-tension caused by the saponin. In the case of ricin lipase, however, saponin caused inhibition, and this, he concluded, was due to a poisoning action on the enzyme.

Exp. No. 44 shows the effect of cholalic acid and its derivatives, choleic and deoxycholeic acids. These substances were preparations made and kindly supplied by Dr S. R. Schryver. The first two produced inhibition, the latter, acceleration.

Exp. No. 44. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Cc. *N*/10 NaOH			
Minutes	Normal	Cholalic acid	Choleic acid	Deoxycholeic acid
5	2·6	1·8	2·4	2·6
10	5·6	3·8	4·8	5·8
15	8·3	5·6	6·4	8·8
20	10·7	7·5	8·1	12·0
25	13·0	9·2	9·6	15·9
30	15·1	10·8	11·1	19·4

That the sensitiveness of the reaction to cholalic acid was as great as to the bile-salts is shown by Exps. 46 to 52 (amount of inhibition in cc. *N*/10 NaOH only given).

Exps. Nos. 45 *to* 51. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Inhibition caused by cholalic acid in concentration of:						
Minutes	2 %	1 %	0·5 %	0·1 %	0·05 %	0·01 %	0·005 %
5	0·2	0·0	0·0	0·0	0·1	0·0	0·0
10	0·3	0·4	0·2	0·1	0·2	0·0	0·0
15	1·1	1·0	0·7	0·6	0·3	0·2	0·2
20	1·7	1·4	1·4	1·0	0·6	0·4	0·3
25	2·6	2·0	1·6	1·4	0·7	0·8	0·4
30	3·3	2·6	2·1	1·7	0·9	1·1	0·6

The effect of saponin was investigated and found to produce slight acceleration in large concentration (3 %, Exp. 52); in weaker concentrations (0·5 %, Exp. 53) little change was noted.

Exps. Nos. 52 *and* 53. *Rabbit's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Cc. *N*/10 NaOH		Cc. *N*/10 NaOH	
Minutes	Normal	Saponin (3 %)	Normal	Saponin (0·5 %)
5	2·6	2·8	2·6	2·7
10	5·8	6·2	5·3	5·2
15	8·8	9·7	8·4	8·4
20	12·1	13·0	11·5	11·4
25	15·1	16·3	14·8	14·6
30	17·9	19·4	17·7	17·4

Cholesterol showed no marked activity in either direction (Nos. 54 and 55).

Exps. Nos. 54 *and* 55. *Cat's Liver.* 10 % *Extract.* Quantities:—As in No. 2.

	Cc. *N*/10 NaOH		Cc. *N*/10 NaOH	
Minutes	Normal	Cholesterol	Normal	Cholesterol
5	2·1	2·2	1·5	1·6
10	4·5	4·7	2·9	3·0
15	7·0	7·1	4·4	4·6
20	9·0	8·9	5·5	5·8
25	10·6	10·5	6·5	7·0
30	12·0	11·9	7·4	8·1

Summary and Conclusions.

1. There has been found to be present as a constant constituent in the bile of all the animals examined, a substance possessing the power of inhibiting the hepatic esterase.

2. Investigation of the action, on the esterase of one animal, of the bile of other species, showed that the inhibitor is probably of the same nature in all biles. At the same time, the results suggest that the concentration of the inhibitor in the bile of a particular animal is that best adapted for producing inhibition of the esterase in that animal.

3. Chemically the inhibiting substance was found to be soluble in alcohol, but insoluble in ether. A solution of Plattner's crystals was found to be as active as the bile in producing inhibition, and, since purification of the crystals by recrystallisation led to no loss of inhibitory power, the inhibitor may be concluded to be the bile-salts.

4. Esterolysis by the hepatic enzyme is extremely sensitive to the presence of bile-salts, as low a concentration as 0·005 % producing inhibition. Since the reaction is also inhibited by cholalic acid and is equally sensitive to this substance, it may be concluded that the bile-salts act as inhibitors by reason of the cholalic acid portion of their molecule.

5. The inhibition does not appear to be in any way due to the physical properties of bile-salts in lowering surface-tension as saponin does not produce a similar result.

6. Cholesterol does not inhibit the action of the esterase.

In conclusion I should like to record my great indebtedness to Prof. E. P. Cathcart for much help and guidance in the prosecution of the research.

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References.

Flohr (1919). *Arch. Néerland. Physiol.*, **3**, 182.
von Fürth and Schütz (1906). *Centralb. Physiol.*, **20**, 47; *Beiträge*, **9**, 28.
Hewlett (1905). *Johns Hopkins Hosp. Bull.*, **16**, 20.
Loevenhart (1906, 1). *J. Biol. Chem.*, **2**, 391.
—— (1906, 2). *J. Biol. Chem.*, **2**, 427.
—— and Souder (1906). *J. Biol. Chem.*, **2**, 415.
Magnus (1904). *Zeitsch. physiol. Chem.*, **42**, 149.
—— (1906). *Zeitsch. physiol. Chem.*, **48**, 376.
Ramond (1904). *Compt. Rend. Soc. Biol.*, **2**, 142.
Rosenheim and Shaw-McKenzie (1910). *J. Physiol.*, **40**. *Proc.* viii, xii.

XXXV. A SERIES OF ABNORMAL LIESEGANG STRATIFICATIONS.

By EMIL HATSCHEK.

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(With Plate VI.)

The experiments described in the present paper represent preliminary work for an investigation in which it was necessary to produce Liesegang stratifications in gelatin specimens having volumes up to 300 cc. and completely submerged in the aqueous solution of the one component. It was therefore desirable to employ a material less expensive than silver nitrate and, for reasons apart from the cost, to produce in the gelatin a precipitate more closely corresponding to some of the mineral constituents of organic structures. I accordingly determined to study the deposition of calcium phosphate in gelatin gel.

The reaction between alkaline phosphates and calcium nitrate has been used by Stephane Leduc [1911]. He used Liesegang's procedure, which consists in placing a drop of the solution of one reaction component on a film of gelatin gel containing the other, and obtained stratifications of such fineness that they acted as a diffraction grating. It has been shown conclusively by Liesegang that this effect is due to wrinkling of the surface of the gel [1913]. This particular effect would have been useless for my purpose, which was to produce stratifications extending through a considerable depth of gel, and I accordingly studied the reaction in test tubes. This procedure is now well known: a test tube is partly filled with the sol containing one of the components in suitable concentration, the sol is allowed to set completely, and the aqueous solution of the other component—which must, in all cases, have a molar concentration greatly in excess of that in the gel—poured on.

After some preliminary experiments, which it is not necessary to describe in detail, it was found that the best results were obtained with gels containing 10 g. of gelatin in 100 cc. of trisodium phosphate solution, the concentration of the latter being 3, 2·5 or 2 % of the crystallised salt ($Na_3PO_4.12H_2O$, *i.e.* respectively 7·87, 6·57 and 5·26 millimoles of Na_3PO_4), on which were placed solutions of calcium chloride, calcium nitrate or mixtures of calcium chloride and sodium chloride. The use of such mixtures, one constituent of which takes no part in the reaction, is a well known device for reducing the concentration of the reacting constituent, while keeping the total molar concentration—which settles the rate of diffusion into the gel—sufficiently high.

In preparing the gelatin sol a somewhat unusual procedure was adopted, except in a few instances, which will be mentioned specially. The usual course—dissolving the salt in the sol—proved objectionable, as all five brands of gelatin used in the investigation gave a, more or less copious, precipitate with trisodium phosphate. If the salt, as is usually done, was added to the sol, the protective effect of the latter rendered the precipitate so fine that the greater portion passed through the Chardin paper usually employed for filtering gelatin. The gelatin leaf was therefore allowed to swell in the phosphate solution itself: in this way whatever precipitate is produced forms in the gelatin gel, which renders it coarser even than it would be in aqueous solution. After 24 hours' swelling the gelatin was dispersed by heating to about 90° and then filtered on a hot water funnel, at about the same temperature, through Chardin paper. Treated in this way all five gelatins gave filtrates as clear as those obtained from the same gelatins without the addition of phosphate. The filtered sol, while still warm, was poured into the test-tubes and allowed to set for about 24 hours before the aqueous solutions were poured on. In some instances, which will be specially referred to, the sol was allowed to set, and the resulting gel re-melted for filling into test-tubes.

The times allowed for swelling, dispersion and gelation in the test-tubes were strictly adhered to throughout, unless a departure from the standard procedure is expressly mentioned; the time required for filtering different brands, which varied considerably, was unfortunately beyond control. As the phosphate solution is markedly alkaline, the difference in the times during which the sols were exposed to the filtering temperature of about 90° (this time varying from 30 to 90 minutes for 200 cc. of different brands) and the consequent difference in hydrolysis may, in part, account for the astonishing divergence in the results obtained with different brands in conditions otherwise alike. It may be well to add that stratifications are obtained only with trisodium phosphate, and not with either Na_2HPO_4 or NaH_2PO_4.

Five brands of gelatin were investigated: (1) a pre-war German table gelatin of unknown origin and moderate "hardness," (2) an English calf gelatin, (3) and (4) two different brands of English gelatin of the same maker, and (5) a French photographic gelatin, the last four brands being all markedly hard. A selection of the most striking results is illustrated in Pl. VI, figs. 1 to 12.

Figs. 1, 2 and 3 show stratifications of the normal type, but of unusual perfection as regards both the regularity of the strata and the entire absence of precipitate in the clear spaces between them. The number of strata is unusually large in No. 3. The preparations were obtained as follows: No. 1, gelatin (2) with 3 % of trisodium phosphate, the aqueous solution consisting of equal parts of 10 % solutions of calcium chloride and sodium chloride; No. 2, gelatin (1) with 2 % of trisodium phosphate, the aqueous solution consisting of four parts of 10 % calcium chloride and one part of 10 % sodium chloride solution; No. 3, gelatin (1) with 2 % of trisodium phosphate, the aqueous solution containing 10 % of crystallised calcium nitrate.

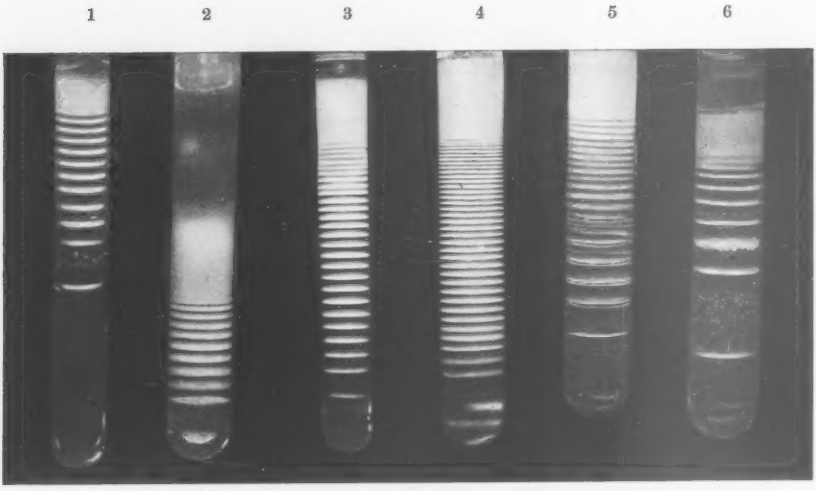
Anomalous features first show themselves in No. 4. The strata are extremely numerous and regular, but are curved, the *upper* side being convex. This is quite unusual: if strata are curved at all, they are concentric with the meniscus of the gelatin gel, *i.e.* the concave side is turned upwards. In addition, many of the strata show a small opening about the centre. This specimen was obtained with gelatin (1), containing 3 % of trisodium phosphate, and an aqueous solution of 20 % of crystallised calcium nitrate.

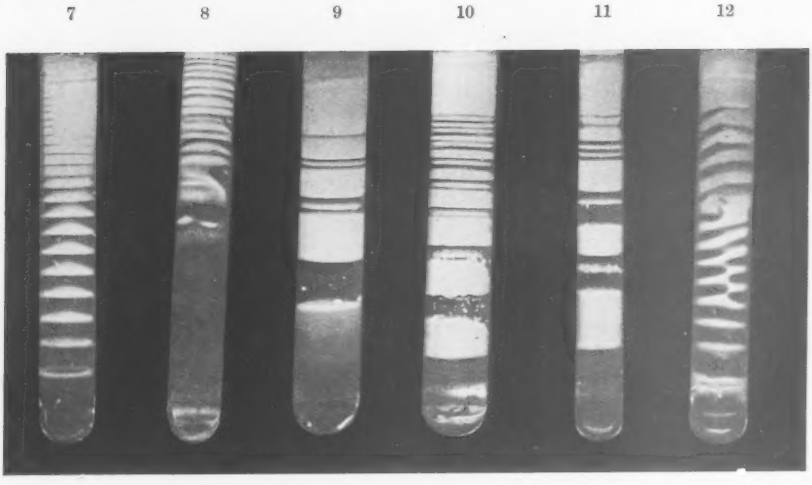
A different anomaly is exhibited by No. 5, gelatin (4), with 3 % trisodium phosphate and a 20 % solution of calcium nitrate. Below the strata there appear rings of precipitate which are confined to the glass surface. As this brand of gelatin exhibited the same tendency with other concentrations of phosphate and other aqueous solutions, its further use was abandoned.

No. 6 is a very remarkable specimen. The gelatin was (2), with 2 % of trisodium phosphate, while the aqueous solution was a mixture of two parts of 10 % $CaCl_2$ and three parts of 10 % NaCl solution. The last four strata alternate with layers of macroscopic aggregates, and the distances, especially between the last two strata, are remarkably great. This alternation between zones containing a large number of small, and a small number of large, particles, has been observed before on a microscopic scale [Hatschek, 1911], but never in the striking fashion exhibited by the present specimen.

Even more striking anomalies are, however, exhibited by Nos. 7 to 12. Some of these appear to have been caused purely by slight (intentional) variations in the procedure adopted in soaking the gelatin etc. Thus in No. 7, the gelatin, phosphate concentration and the aqueous solution were exactly the same as used for No. 3. The gelatin was, however, soaked in water for 30 minutes only, dispersed by warming and the trisodium phosphate added to the sol, which was then filtered. The strata, although spaced normally, show a very marked convexity upwards, and a tendency to the formation of central stems. Similar convex strata are shown by No. 8, but these are arranged quite regularly in pairs. In this case the gelatin and the phosphate concentration were the same as in No. 1, but an aqueous solution of 20 % $Ca(NO_3)_2.4H_2O$ was used. Nos. 9, 10 and 11 show an even more striking feature not observed hitherto: the formation of wide and narrow bands of precipitate, which alternate regularly in No. 9 and—apparently—quite erratically in Nos. 10 and 11. In these three cases the gelatin was soaked for only 45 minutes: Nos. 9 and 11 were gelatin (2) with 2 % of trisodium phosphate, the aqueous solution being respectively 10 % $Ca(NO_3)_2.4H_2O$ and 7·5 % $CaCl_2$. No. 10 contained 2·5 % of trisodium phosphate, the brand of gelatin and the aqueous solution being the same as used in No. 9. The gel used for No. 9 had been re-melted about 24 hours after filtration.

No. 12 finally shows the result obtained in a gelatin gel *hardened by formaldehyde before* the solution of the calcium salt was allowed to diffuse into it. The gel was gelatin (1) with 2 % trisodium phosphate, which was hardened by allowing a solution containing 1 % formaldehyde and 2 %


1
2
3
4
5
6


7
8
9
10
11
12

trisodium phosphate to diffuse into it for about five days. The addition of phosphate to the formaldehyde solution is of course necessary to prevent the salt from diffusing out of the gel. After five days the formaldehyde solution was poured off and was replaced by a 10 % solution of $CaCl_2$. The curious pseudo-helical arrangement has never, to my knowledge, been observed before in test-tube experiments: it recalls, however, the spirals obtained by Liesegang [1914], with the silver chromate reaction carried out in his usual manner on plates, in gelatin gels of suitable acid and gelatose content.

In the present unsatisfactory state of our knowledge regarding the normal phenomenon it is impossible even to attempt an explanation of the extremely complicated structures just described. Other reactions, such as Liesegang's original example, are also sufficiently sensitive to small variations in the brand of gelatin, its acid and gelatose content, in the concentration of the reacting solutions and in the procedure adopted. The resulting stratifications differ, however, generally speaking, in number, in the width of the chromate bands and of the spaces separating them and in the amount of diffuse precipitate found in the latter: such anomalies as bands distinctly grouped in pairs, or alternately wide and narrow, have never been described. While the results therefore enormously increase the difficulty—sufficiently great even in view of the material published hitherto—of propounding a general theory of the phenomenon, they may also enhance its importance to the biologist and geologist by showing that structures of a much more complicated nature than any hitherto obtained in such experiments may be the result of simple diffusion into a gel.

REFERENCES.

Hatschek (1911). *J. Soc. Chem. Ind.*, No. 5, **30**.
Stephane Leduc (1911). *Theorie physico-chimique de la vie*, Fig. 16.
Liesegang (1913). *Kolloid. Zeitsch.*, **12**, 181.
—— (1914). *Zeitsch. physikal. Chem.*, **88**, 1.

XXXVI. THE ACIDITY OF ROPY MILK.

By KATHLEEN FREEAR

AND

ELFRIDA CONSTANCE VICTORIA VENN.

From the Research Institute in Dairying, University College, Reading.

(*Received March 25th, 1920.*)

Ropy Milk.

In 1916 two samples of ropy milk from two Lancashire farms were sent to the laboratory by Miss Stubbs, Lancashire County Council Dairy School, Hutton.

The samples were plated out upon neutral lemco agar and from both of them a gram-positive *Coccus* showing also diplococcal forms, and in fluid cultures short chains, was obtained. Sometimes the cocci appeared lanceolate and possibly capsulated, the latter appearance being most marked in glucose broth cultures. The cultures which were obtained were called *Bury* and *Accrington* since the farms from which the samples came were in the neighbourhoods of these towns.

After satisfactory isolation had been carried out the bacteriological characteristics of two strains B_{12} and B_{15} from the Accrington farm and two others RM_4 and RM_5 from the Bury farm were studied. The results obtained appeared to show that these organisms were either identical with or closely related to the *Streptococcus Hollandicus*, and they were used as starters with satisfactory results in the preparation of Edam cheese.

For purposes of comparison strains of *Streptococcus Hollandicus* were obtained from Prof. Orla Jensen and also from Hoorn and Delft. The reactions of these strains were studied upon solutions of sugars, alcohols, etc. which were prepared in the following way. Tubes of media containing 1 % peptone and 0·5 % NaCl were neutralised to litmus; they were then coloured with litmus and 0·5 % of the "sugar" was added except in the cases of inositol, sorbitol and dulcitol, of which only 0·25 % was used. The tubes were then sterilised by steaming on three consecutive days. The reactions produced by the various strains upon these media are shown in Table I. It does not appear that the strains which had been isolated from the milk obtained from Accrington and Bury differed in any material respect from those found with the strains of *Streptococcus Hollandicus*, especially when the method of preparation of the media, and the difficulty of obtaining constant reactions with streptococci upon such media are considered.

Table I.

Ropy Milk.
February 21, 1917.

	Accrington	Bury	Hoorn	Delft	Jensen
Saccharose	—	Sl. acid	—	—	?
Mannitol	Acid	Acid	—	—	V. sl. acid
Galactose	Acid	Acid	Acid	—	—
Adonitol	—?	—	—	—	?
Dextrose	Acid	Acid	Acid	Sl. acid	?
Glycerol	Sl. acid?	—	—	—	?
Dextrin	Acid	Acid	Acid	—	—
Raffinose	—	—	—	—	—
Lactose	Acid	Acid	Acid	—	—
Inulin	Sl. acid?	V. sl. acid?	—	—	?
Sorbitol	—?	—	?	—	—
Inositol	—?	—	V. sl. acid	?	Sl. acid
Dulcitol	—?	Acid	—	?	?
Salicin	Acid	V. sl. acid?	Acid	—	Acid?

While the bacteriological examinations of the strains were being carried out an occasional determination of the acidity of samples of the ropy milk was made, and was always found to fall within narrow limits. It was therefore decided to study the range of acidity within which the isolated strains produced ropiness and with this object the following experiments were carried out.

The milk under investigation was said to be "Ropy," when definite threads could be drawn out from its surface by means of a sterile platinum needle. Under the conditions of experiment very long ropy threads were not obtained and the ropiness was considered to be "*good*" when the length of the threads approximated to an inch. When the threads were considerably shorter than this but nevertheless distinguishable from mere stickiness, the ropiness was described as "slight." During the last series of determinations the degree of ropiness was further estimated by means of simple viscosity determinations. The method was not sufficiently accurate to justify the inclusion of the results in the text, but it may be mentioned that simultaneously with the appearance of "good rope" there was a sudden and very marked increase in viscosity. With the diminution of "ropiness" there was a corresponding decrease in viscosity, until with its disappearance the viscosity value obtained closely approximated to that of the uninoculated milk.

In the first series of experiments, the results of which are shown in Fig. 1, flasks of sterile milk, which had been inoculated with the strains isolated from the samples of ropy milk, were incubated at 22°. Samples were withdrawn, tests for ropiness made, and determinations of acidity with $N/9$ NaOH using phenolphthalein as indicator were carried out every 24 hours for a period of 7 days. Fig. 1 shows that ropiness was found on the first day after inoculation except in the case of B_{15} when it did not appear until the second day, and that it occurred between acidities which varied between 0·44 and 0·75 (% lactic acid calculated from titrations with $N/9$ NaOH). Ropiness was well marked

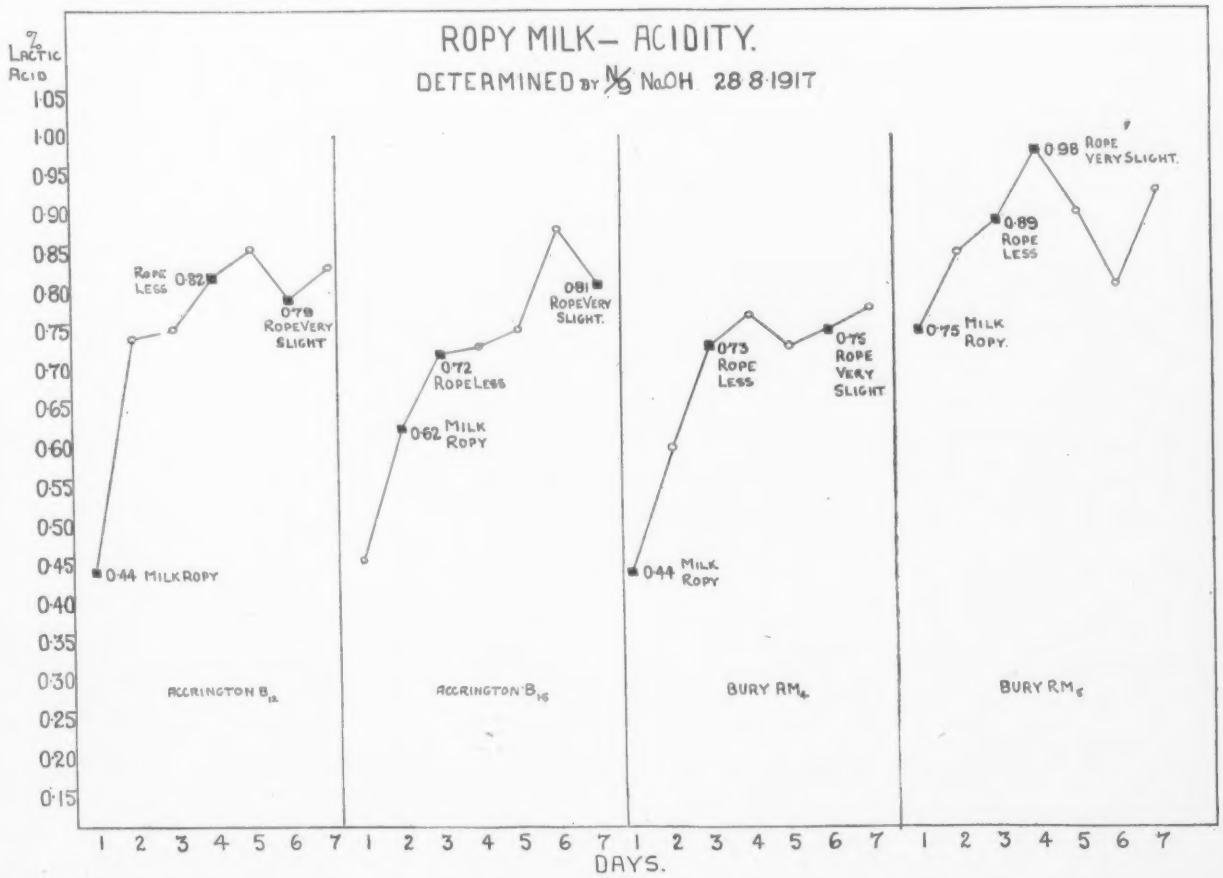

ROPY MILK— ACIDITY.
DETERMINED BY N/9 NaOH 28.8.1917
% LACTIC ACID
1·05
1·00
0·95
0·90
0·85
0·80
0·75
0·70
0·65
0·60
0·55
0·50
0·45
0·40
0·35
0·30
0·25
0·20
0·15
ROPE LESS 0·82
0·78 ROPE VERY SLIGHT
0·44 MILK ROPY
ACCRINGTON B12
0·72 ROPE LESS
0·62 MILK ROPY
0·81 ROPE VERY SLIGHT.
ACCRINGTON B16
0·73 ROPE LESS
0·75 ROPE VERY SLIGHT
0·44 MILK ROPY
BURY RM4
0·98 ROPE VERY SLIGHT.
0·89 ROPE LESS
0·75 MILK ROPY.
BURY RM6
1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7 1 2 3 4 5 6 7
DAYS.

Fig. 1.

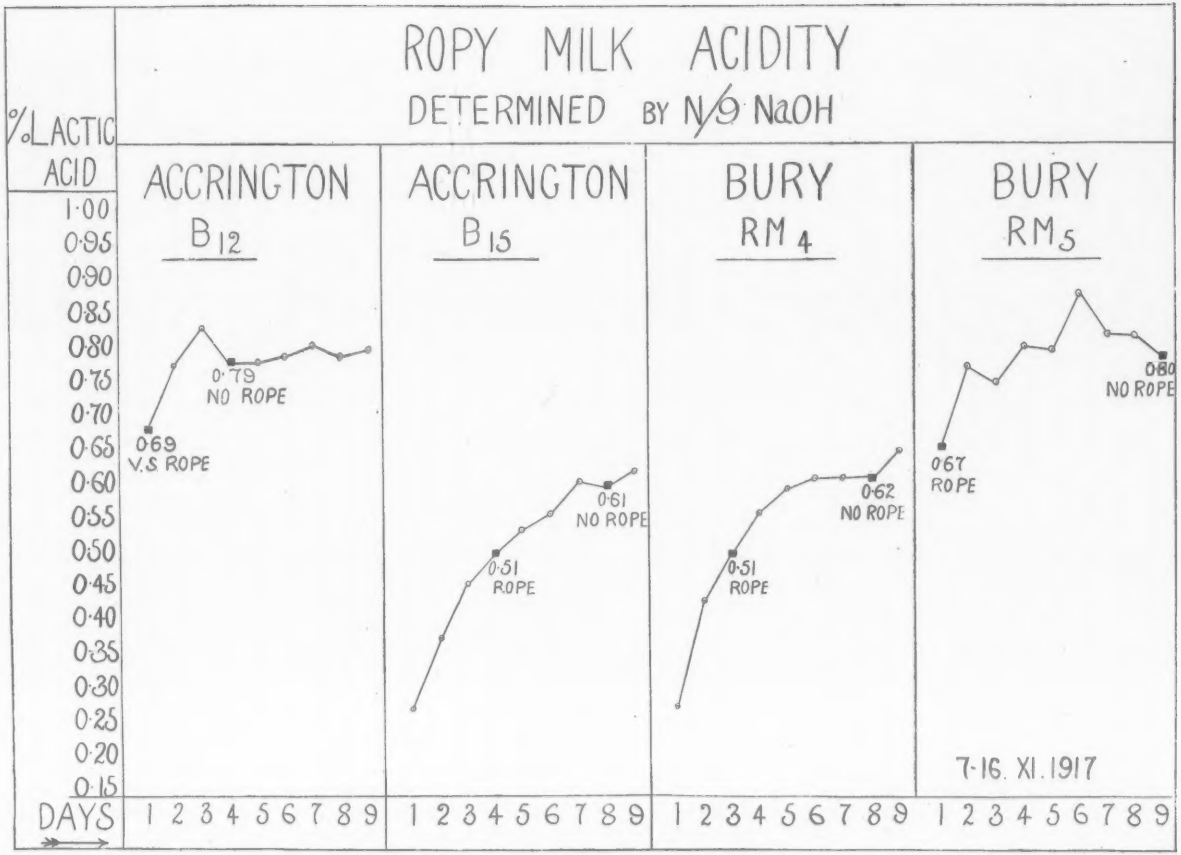

ROPY MILK ACIDITY
DETERMINED BY N/9 NaOH
% LACTIC ACID
1·00
0·95
0·90
0·85
0·80
0·75
0·70
0·65
0·60
0·55
0·50
0·45
0·40
0·35
0·30
0·25
0·20
0·15
ACCRINGTON B12
0·69 V.S ROPE
0·79 NO ROPE
ACCRINGTON B15
0·51 ROPE
0·61 NO ROPE
BURY RM4
0·51 ROPE
0·62 NO ROPE
BURY RM5
0·67 ROPE
0·80 NO ROPE
7·16. XI. 1917
DAYS
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9

Fig. 2.

for the first three or four days, at the end of which time the acidities varied between 0·72 and 0·89; subsequently the ropiness diminished without any marked increase of acidity, but was still present to a very slight extent at the end of the experiments. A second series of experiments was then undertaken, partly to confirm the results already obtained, and partly to prolong the duration and, if possible, to find the period at which the ropiness really disappeared. Fig. 2 shows that sometimes ropiness did not appear until the fourth day after inoculation. It continued to be present in gradually diminishing amount for periods which varied from three to eight days. The range of acidity over which ropiness was present was 0·51 to 0·87. At the end of the experiments the ropiness had disappeared, though no appreciable alteration in the acidity had taken place.

In continuation of this series two strains from each of the cultures which were under investigation were studied and with them two controls of sterile inoculated milk. Fig. 3 shows that the acidities of the controls lay within the values 0·17 and 0·20. In all the inoculated flasks ropiness was found at the end of 24 hours, the acidity varying at this time from 0·59 to 0·82. The ropiness remained well marked for periods which varied from one to three days with acidities which lay between 0·59 and 0·96 except upon one occasion when the acidity was found to be 1·435. It is doubtful whether this reading does not represent an experimental error. The ropiness began to diminish from two to four days after inoculation and became gradually less until it ceased to be present on the sixth to the ninth day. In all cases the ropiness had disappeared at the end of the experiment which was carried out over a period of nine days. The diminution of ropiness was accompanied by an acidity which varied between 0·83 and 1·0. The disappearance of ropiness was not accompanied by any material variation in the acidity.

In these experiments the examinations of the samples were first made 24 hours after inoculation of the milk, by which time the ropiness was in many cases well established. In all later experiments, therefore, the inoculations were made at midnight and the milk was tested at six hour intervals for the first 18 hours, and afterwards at intervals of 12 hours. The third series of experiments was carried out under these conditions and Fig. 4 shows that at the 30th hour after inoculation the acidity was 0·30 and no ropiness was found, but at the 42nd hour the acidity was 0·73 with fair ropiness. At the end of another 12 hours, when the acidity was 0·9 the ropiness was already decreasing, and continued to decrease until it had completely disappeared 102 hours after the inoculation when the acidity was 0·97. The highest acidity recorded during the period of the experiment was 1·00.

It was thought that the initial acidity at which ropiness occurred might be fixed with greater accuracy if more continuous determinations were made, but it was not possible to carry out satisfactory titration experiments at night. In the later experiments, therefore, the acidity determinations were made not only by titrating with $N/9$ NaOH but also electrometrically.

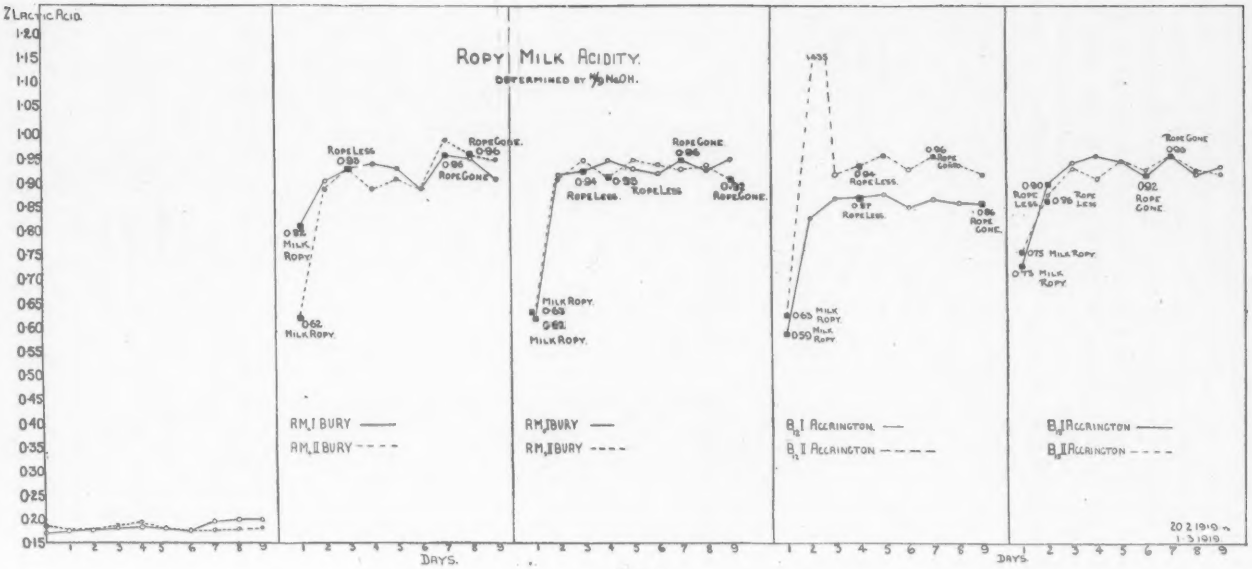

% Lactic Acid.
Ropy Milk Acidity.
Determined by N/9 NaOH.
Rope Less
Rope Gone
Milk Ropy
Rope Less.
Rope Gone.
Milk Ropy.
RM₁ I Bury
RM₁ II Bury
RM₂ I Bury
B₁₂ I Accrington
B₁₁ I Accrington
B₁₁ I Accrington
Days.
20 2 1910 to 1 3 1910

Fig. 3.

Figs. 5 and 6 show the results obtained by both methods in the course of the next experiments. It is seen that ropiness was present at the 18th hour after inoculation, when the percentage of lactic acid was 0·43 and the P_H was 5·0. It continued to be well marked for 102 hours, when it was found to be diminishing. During this period the acidity as determined by $N/9$ NaOH rose from 0·43 to 0·81, and the P_H changed from 5·0 to 4·0. After the 102nd hour the ropiness diminished, while the acidity remained practically unchanged. The quantity of milk available was insufficient to carry on the

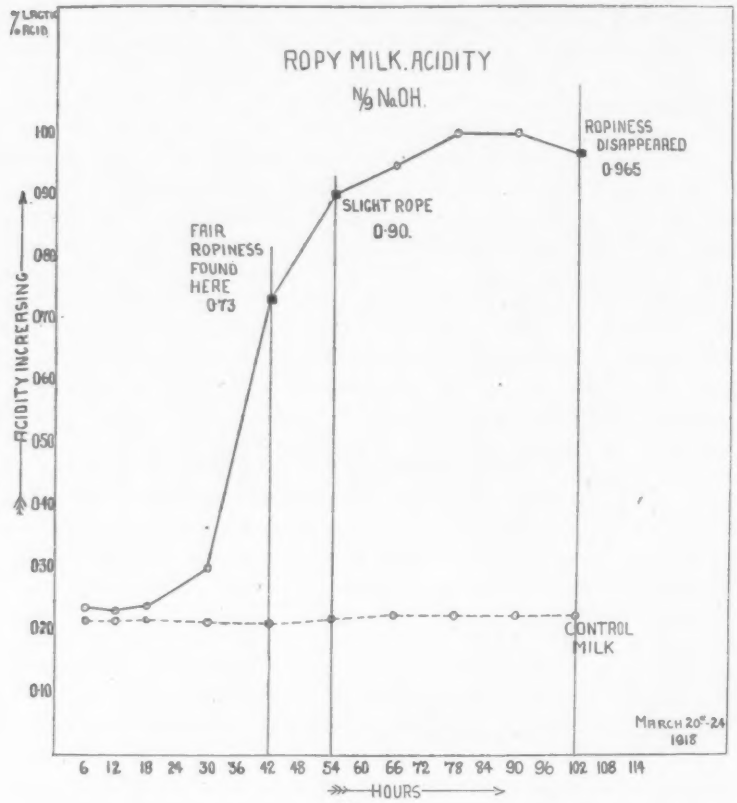


Fig. 4.

experiment beyond 138 hours, and it was therefore repeated with a much larger bulk of milk. In order to ensure sterility of such a volume the heating was necessarily prolonged with the result that a certain amount of charring took place, rendering the end point of the titrations with $N/9$ NaOH indeterminate, for which reason these readings are not included. The results of the electrometric determinations are shown in Fig. 7 from which it appears

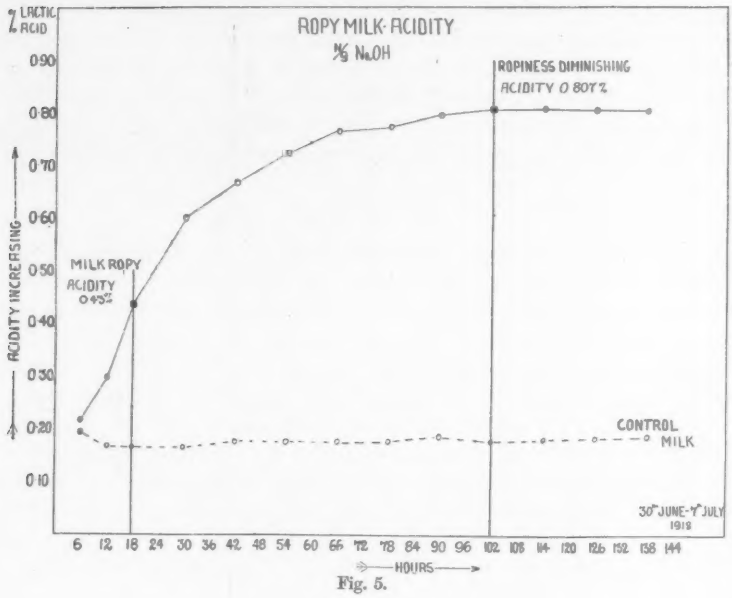

% LACTIC ACID
ROPY MILK ACIDITY
N/9 NaOH
ROPINESS DIMINISHING
ACIDITY 0·80%
0·90
0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
ACIDITY INCREASING
MILK ROPY
ACIDITY
0·43%
CONTROL
MILK
30th JUNE-1st JULY
1918
6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96 102 108 114 120 126 132 138 144
HOURS

Fig. 5.

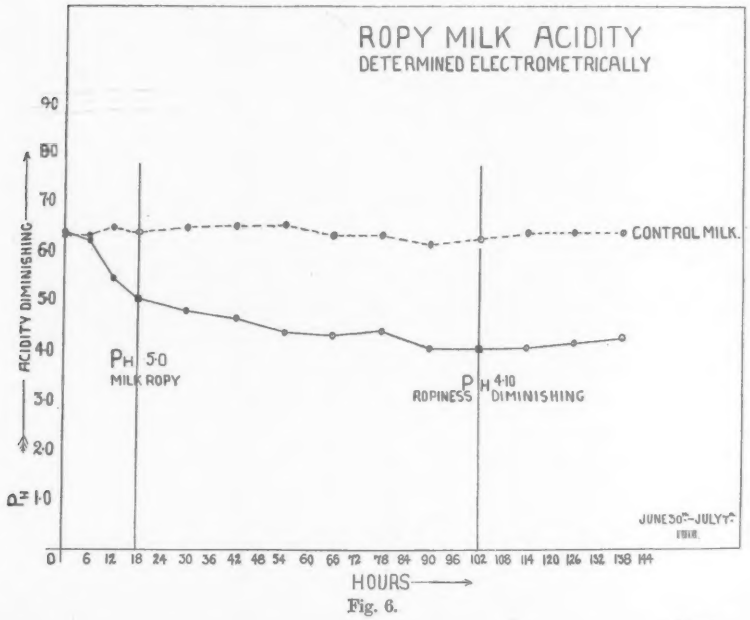

ROPY MILK ACIDITY
DETERMINED ELECTROMETRICALLY
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
ACIDITY DIMINISHING
PH
CONTROL MILK.
PH 5·0
MILK ROPY
PH 4·10
ROPINESS DIMINISHING
JUNE 30th-JULY 1st
1918.
6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96 102 108 114 120 126 132 138 144
HOURS

Fig. 6.

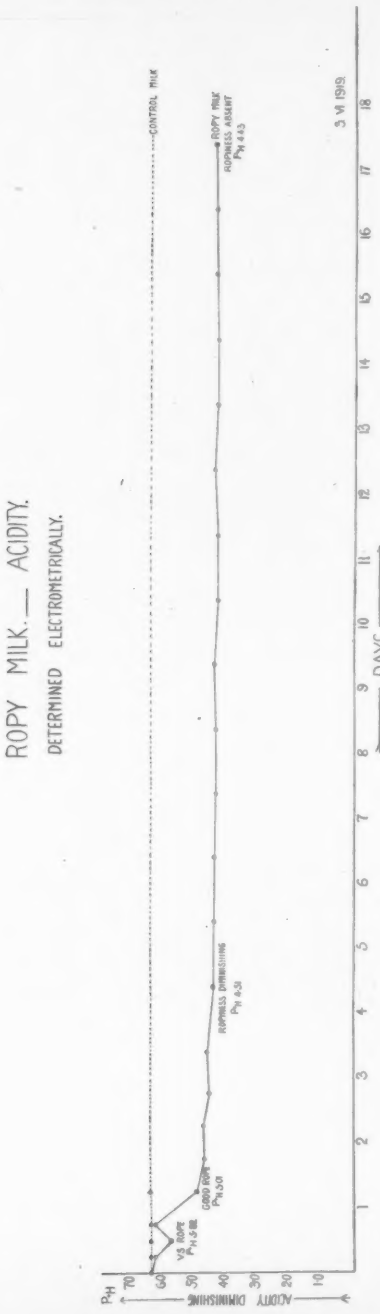

ROPY MILK.— ACIDITY.
DETERMINED ELECTROMETRICALLY.
CONTROL MILK
ROPY MILK
ROPINESS ABSENT
PH 4.45
3 VI 1919
VS. ROPE
PH 5.98
GOOD ROPE
PH 5.30
ROPINESS DIMINISHING
PH 4.58
PH
ACIDITY DIMINISHING
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
DAYS

Fig. 7.

that slight ropiness was first found at the 12th hour after inoculation when the P_H was 5·82. At the 30th hour good ropiness had developed and the P_H was 5·01. Satisfactory ropiness continued for three days at the end of which time the P_H was 4·51. The ropiness then gradually diminished until, 17 days after the inoculation, it had entirely disappeared, but throughout this period the acidity remained practically unchanged.

Conclusions.

The experiments show that these strains produced ropiness in milk, associated with a minimum acidity, as determined by *N*/9 NaOH, of 0·43 % lactic acid, and that the ropiness continued for varying periods of time, accompanied by an acidity which never rose above 1·0 % (except on one occasion). In every case, the ropiness eventually diminished and when studied for sufficiently long periods entirely disappeared, but these changes were not accompanied by any material alteration in the acidity.

The results obtained by the NaOH titration method were confirmed by electrometric determinations, from which it appeared that ropiness was found in milk with a P_H range of 5·82 to 4·10. With diminishing ropiness no appreciable alteration in the P_H value could be detected, although in one case the experiment was continued for 17 days by which time ropiness had disappeared.

XXXVII. THE OCCURRENCE AND NATURE OF THE PLANT GROWTH-PROMOTING SUBSTANCES IN VARIOUS ORGANIC MANURIAL COMPOSTS.

By FLORENCE ANNIE MOCKERIDGE.

From the Department of Botany, King's College, London.

(*Received March 29th, 1920.*)

The generally accepted idea of the nutrition of an ordinary autotrophic plant is that it requires only a supply of inorganic food materials, consisting of minerals, carbon dioxide and water, from which it is able to elaborate the whole of its organic substance. This conception of plant nutrition, which forms one of the main physiological distinctions between plant and animal, has been modified during recent years by the work of Bottomley, in which he has been assisted by the writer. Experimenting on the growth of green plants of *Lemna minor* in water culture, he has found [Bottomley, 1917, 1] that the addition to the inorganic nutrients of minimal quantities of certain organic substances is absolutely essential if the plants are to grow healthily and normally for any length of time. These organic substances, which have been termed "auximones" by Bottomley, are apparently similar in function, though not necessarily so in nature, to the "vitamines," the accessory growth substances, which play such an important part in animal nutrition.

The use of water-cultures affords practically the only means of absolutely controlling the food materials supplied to a plant, and water plants are obviously the best suited to such growth, since they normally grow with their roots submerged. This fact, together with their rapid multiplication by budding, afforded the reason for the choice of *Lemna minor* for the experiment; and the result of the experiments led to the suggestion that perhaps this plant was peculiar in requiring some organic addition to its nutrient solution. A number of other water-plants have since been tested in the same way, including *Lemna major*, *Salvinia natans*, *Azolla filiculoides*, and *Limnobium stoloniferum*, and these have all been found to grow healthily and multiply rapidly in the presence of a small quantity of organic material, while in inorganic materials alone they gradually died off [Bottomley, 1920, 2], and this was the case no matter what the nutrient solution employed [Bottomley, 1920, 1]. The well-known fact that ordinary plants will grow well in water culture is explained by the comparatively large quantity of

organic food introduced in their seed, and it was shown some time ago [Bottomley, 1914] that wheat seedlings with their endosperm removed at a very early stage responded very much more quickly to the addition of these organic materials than plants which were not so treated. A number of experiments, which will be reported in due course, have more recently been carried out with cuttings; and these also demonstrate the absolute necessity of organic materials for normal healthy growth, and explain the prevalent idea of the unsuitability of cuttings for water culture. All these experiments tend to the conclusion that all green plants require the presence of organic material of a special kind for their maximum growth and development.

An entirely different type of plant, a *Saccharomyces*, has recently been the subject of similar researches on the part of Williams [1919] and Bachmann [1919]. Williams arrives at the conclusion that "a substance of unknown nature...is necessary in addition to the ordinary nutrients for the nutrition of yeast cells," and the effect of this substance is so marked that he suggests that yeast may be used as a biological test for vitamines. He also concludes that this substance is identical with the animal beri-beri vitamine. Bachmann finds that while the various races of yeast vary in their vitamine requirements, yet they all grow better and ferment more readily in a medium containing small amounts of some organic material in addition to their carbohydrate. This necessary substance appears to be a constituent of the yeast cell, and is presumably a synthetic product of the organism, which is therefore apparently stimulated by the products of its own growth.

That all of the animal vitamines are direct products of plant synthesis appears to be probable. It has been ascertained by numerous workers that animals obtain their necessary vitamines from their plant food. It is now established that plants, in their turn, require growth-promoting substances, or auximones; which, in the case of some of the lower plants, are apparently manufactured by themselves, but which in the case of green plants, must be supplied from without. Since these necessary accessory substances are essentially organic in nature, their only possible source in the case of ordinary green plants is to be found in the organic matter of the soil in which they are growing.

Bottomley [1914 and 1917, 1] has found that the auximones are produced very largely during the bacterial decomposition of peat by aerobic organisms, and that they are water-soluble, being extracted by water from peat so treated. The present writer [Mockeridge, 1917] has shown that the auximones thus extracted stimulate not only plant growth, but also the activity of the soil organisms, especially of those concerned in the bacterial treatment of the material; an observation which suggests that the bacteria concerned are stimulated by the products of their own growth, and brings them into line in this respect with the yeasts. Bottomley [1919] has found that the products of the nitrogen-fixing bacteria, *Azotobacter chroococcum* and *Bacillus radicicola*, grown on pure synthetic media, can function as growth-promoting substances

for the higher plants. All of these observations tend to relegate the ultimate production of growth-promoting substances, whether plant or animal, to the soil organisms, and suggest that the moulds and bacteria of the soil, growing upon the organic matter, produce these necessary substances, which are then absorbed by the plants and utilised in their metabolism, and eventually passed on, either in the same form in which they were originally absorbed, or as some other product of plant synthesis, to the animal in its plant food.

If this suggestion be correct, then the greater the bacterial activity in the soil, the greater should be its supply of plant auximones; and the bacterial activity may be concluded to be dependent upon the amount of organic matter present. Consequently, the value of the ordinary organic manurial materials which are necessary for plant culture, and the rôle of which has given rise to so much speculation, may be due, at least partially, to the presence of growth-promoting substances which have been set free by bacterial action; for the artificial treatment of such a material as peat with various soil organisms under controlled conditions of temperature is only a hastening of the natural processes which are being carried out in these manurial composts.

Culture Experiments.

A preliminary experiment was therefore carried out to ascertain whether ordinary organic composts contain water-soluble ingredients which are capable of stimulating plant growth. Samples of both fresh and well-rotted stable manures, leaf-mould, and a good well-manured garden soil were obtained. An estimation of the moisture of each sample was made, and the bulk of each material was shaken with distilled water in a shaking machine for several hours, after which the material was filtered through paper in a Buchner funnel by the aid of a pump, and the residue again shaken with distilled water. This process was repeated until all the water-soluble material had been extracted, when all the filtrates were combined, and the volume of the whole liquid measured. A little chloroform was then added to prevent bacterial action, and the whole was preserved in a stoppered bottle until ready for use.

Each of the composts was extracted in the same way, and a water extract of known strength of each material was thus obtained. The effect of these liquids on the growth of *Lemna major* in water culture was then investigated, and compared with that of bacterised peat.

Twenty flat glass crystallising dishes of 500 cc. capacity were arranged in four series of five dishes each. 250 cc. of Knop's nutrient solution (containing potassium nitrate, potassium dihydrogen phosphate, magnesium sulphate, calcium nitrate, and a trace of ferric chloride) were put into each dish, with the following additions: Series I, numbered from 1 to 5, no addition (control series); Series II, numbered from 6 to 10, the water extract of 1 g. of leaf-mould in every 500 cc.; Series III, numbered from 11 to 15, the water extract of 1 g. of soil in every 50 cc.; and Series IV, numbered from 16 to 20, the water extract of 1 g. of bacterised peat in every 1000 cc. These varying proportions of the different materials were chosen because it was anticipated that their effect would probably be in proportion to their water-soluble organic content, and so the quantities supplied were smaller in proportion as the material appeared to be richer

in organic matter. The dishes were covered to the level of the liquids with paper which was black on the inner surface and white on the outer, to prevent the entry of light through the sides and bottom of the dishes.

Ten similar plants of *Lemna major* were counted out into each dish, and 100 similar plants were counted out for an estimation of their dry weight. The dishes were placed in a well-lighted greenhouse, and a sheet of glass was supported over them in order to exclude dust as much as possible. The solutions were changed twice weekly, to prevent bacterial contamination as much as possible, and the plants were counted once each week. The figures obtained for five weeks were as follows:

Table I.

Series No.	Dish No.	Number of plants: At beginning	1st week	2nd week	3rd week	4th week	5th week
I. Knop's Solution	1	10	20	23	23	24	25
	2	10	23	24	26	26	28
	3	10	22	23	26	27	28
	4	10	18	23	27	27	27
	5	10	22	24	24	26	27
	Mean...	10	21·0	23·4	25·2	26·0	27·0
II. Knop's Solution + leaf-mould extract	6	10	24	27	34	43	64
	7	10	27	32	42	52	65
	8	10	25	27	34	43	57
	9	10	26	30	41	50	64
	10	10	23	29	41	53	73
	Mean...	10	25·0	29·0	38·4	48·2	64·6
III. Knop's Solution + soil extract	11	10	27	36	47	53	67
	12	10	23	32	48	54	71
	13	10	21	29	42	45	61
	14	10	26	39	51	53	75
	15	10	25	36	41	47	58
	Mean...	10	24·4	34·4	45·8	50·4	66·4
IV. Knop's Solution + bact. peat extract	16	10	31	43	65	86	142
	17	10	29	40	63	87	132
	18	10	29	44	65	82	130
	19	10	28	38	59	75	118
	20	10	27	44	66	86	141
	Mean...	10	28·8	41·8	63·6	83·2	132·6

At the end of the fifth week the plants in the two dishes of each series which were nearest the mean were thoroughly washed, and an estimation was made of their dry weight. The following figures were obtained:

Average weight of 100 plants in all series at beginning of experiment						33·4 mg.
,,	,,	,, Series	I	at end of experiment		25·3 mg.
,,	,,	,,	II	,,	,,	37·4 mg.
,,	,,	,,	III	,,	,,	38·9 mg.
,,	,,	,,	IV	,,	,,	39·2 mg.

From these figures it is clear that both the extract of soil and that of leaf-mould have the power of increasing the rate of growth of plants of *Lemna major* in water-culture, and also of maintaining them in health; for

while the control plants which were successively budded off from the original ten gradually diminished in size and became sickly and yellowish, the plants in all the other series remained green, and not only maintained their original weight, but increased upon it. Both the soil and the leaf-mould thus contain substances which can be regarded as growth-promoting, since they enable the plants better to assimilate the food materials supplied to them; although, as may be expected, neither the soil nor the leaf-mould, even in the larger quantities used, contain these substances in the comparatively large proportion in which they occur in the compost in which bacterial action has been hastened.

The effect of an extract of stable manure was tested in a separate series of experiments. Samples of both fresh and well-rotted stable manure were obtained, and after an estimation of their moisture content, the materials were thoroughly extracted with water as described above for the other organic composts. A little chloroform was added to the standardised extracts, and this was driven off on the water-bath at about 80° when the liquids were required for use. In all cases the extracts were standardised on the basis of the dry weight of the compost.

Fifty dishes, each containing 250 cc. of Knop's solution, were arranged in ten series of five dishes each.

To these the following additions were made: Series I, numbered from 1 to 5, no addition (control series); Series II, numbered from 6 to 10, a water extract of 1 g. of bacterised peat per 1000 cc.; Series III, numbered from 11 to 15, the water extract of 1 g. of bacterised peat per 2000 cc.; Series IV, numbered from 16 to 20, the water extract of 1 g. of bacterised peat per 5000 cc.; Series V, VI and VII, numbered from 21 to 25, from 26 to 30, and from 31 to 35 respectively, similar proportions of extracts of fresh stable manure; and Series VIII, IX and X, numbered from 36 to 40, 41 to 45, and 46 to 50 respectively, an extract of well-rotted stable manure in similar quantity. Ten plants of *Lemna major* were counted out into each dish, and 100 similar plants were counted out for an estimation of their dry weight. The whole set was treated precisely as in the preceding experiment, the plants being counted once weekly for five weeks. The following results were obtained:

Table II.

Series No.	Dish No.	Number of plants: At beginning	1st week	2nd week	3rd week	4th week	5th week
I. Knop's Solution	1	10	25	31	40	52	58
	2	10	25	32	40	50	53
	3	10	22	28	36	41	43
	4	10	24	27	34	40	45
	5	10	22	30	39	47	49
	Mean...	10	23·6	29·6	37·8	46·0	49·6
II. Knop's Solution + extract 1 gm. bact. peat per 1000 cc.	6	10	31	50	72	115	151
	7	10	31	45	62	102	131
	8	10	30	48	75	118	154
	9	10	29	47	70	110	150
	10	10	27	43	60	100	134
	Mean...	10	29·6	46·6	67·8	109·0	144·0

Table II *continued.*

Series No.	Dish No.	Number of plants: At beginning	1st week	2nd week	3rd week	4th week	5th week
III. Knop's Solution + extract 1 gm. bact. peat per 2000 cc.	11	10	25	41	50	81	91
	12	10	24	40	52	80	91
	13	10	29	42	57	82	100
	14	10	31	43	61	93	105
	15	10	30	43	61	93	108
	Mean...	10	27·8	41·8	56·2	85·8	99·0
IV. Knop's Solution + extract 1 gm. bact. peat per 5000 cc.	16	10	24	33	50	73	85
	17	10	28	40	56	74	91
	18	10	24	38	50	72	89
	19	10	26	40	51	73	83
	20	10	25	35	50	68	80
	Mean...	10	25·4	37·6	51·4	71·8	85·6
V. Knop's Solution + extract 1 gm. fresh manure per 1000 cc.	21	10	24	33	45	54	61
	22	10	23	34	45	55	60
	23	10	20	36	48	58	64
	24	10	24	32	40	50	57
	25	10	24	34	43	51	57
	Mean...	10	23·0	33·8	44·1	53·6	59·8
VI. Knop's Solution + extract 1 gm. fresh manure per 2000 cc.	26	10	26	36	44	55	61
	27	10	22	29	37	49	56
	28	10	21	31	38	49	54
	29	10	22	30	41	52	56
	30	10	24	30	40	54	57
	Mean...	10	23·0	31·2	40·0	51·8	56·8
VII. Knop's Solution + extract 1 gm. fresh manure per 5000 cc.	31	10	25	35	41	54	56
	32	10	22	29	34	47	52
	33	10	23	30	36	50	55
	34	10	21	31	36	46	50
	35	10	22	33	38	45	49
	Mean...	10	22·6	31·6	37·0	48·4	52·4
VIII. Knop's Solution + extract 1 gm. rotted manure per 1000 cc.	36	10	25	42	51	74	82
	37	10	26	36	45	71	81
	38	10	29	41	52	74	84
	39	10	26	40	54	71	77
	40	10	25	37	45	66	72
	Mean...	10	26·2	39·2	49·4	71·2	79·2
IX. Knop's Solution + extract 1 gm. rotted manure per 2000 cc.	41	10	25	37	45	60	76
	42	10	28	38	52	70	75
	43	10	27	38	44	62	72
	44	10	28	41	50	70	78
	45	10	26	36	44	61	72
	Mean...	10	26·8	38·0	47·0	64·6	74·6
X. Knop's Solution + extract 1 gm. rotted manure per 5000 cc.	46	10	25	38	49	58	64
	47	10	26	38	50	67	74
	48	10	26	35	40	58	61
	49	10	29	38	40	57	61
	50	10	28	41	46	60	63
	Mean...	10	26·8	38·0	45·0	60·0	64·6

At the conclusion of the experiment an estimation was made of the dry weight of the contents of the two dishes which were nearest to the mean in each series, and the dry weight of 100 plants in each series was calculated from the figures so obtained. These, compared with the original weights, gave the following results:

Weight of 100 plants in each series at beginning of experiment						35·8 mg.
,,	,,	Series	I	at end of experiment		26·4 ,,
,,	,,	,,	II	,,	,,	52·8 ,,
,,	,,	,,	III	,,	,,	50·4 ,,
,,	,,	,,	IV	,,	,,	51·3 ,,
,,	,,	,,	V	,,	,,	36·6 ,,
,,	,,	,,	VI	,,	,,	36·8 ,,
,,	,,	,,	VII	,,	,,	35·7 ,,
,,	,,	,,	VIII	,,	,,	44·9 ,,
,,	,,	,,	IX	,,	,,	45·3 ,,
,,	,,	,,	X	,,	,,	43·7 ,,

The control plants, as in all previous experiments, had thus not only multiplied far less rapidly, but had also failed to maintain their normal size, as indicated by their weight; while in all the series containing the added extracts, in addition to showing a more rapid rate of multiplication, the plants had maintained their normal health, and even increased in size. It is noteworthy that in all the three proportions in which the extracts were used, the well-rotted stable manure was more effective than the fresh manure, thus indicating that production of growth-promoting substances is more or less proportional to the bacterial decomposition of the material.

The experiments thus far recorded point to the conclusion that all the materials examined, viz. fresh and well-rotted stable manure, leaf-mould, and well-manured soil, as well as bacterised peat, contain water-soluble substances which are capable of functioning as plant growth-promoting substances, or auximones, and the greater the bacterial decomposition of the organic matter of the compost, the greater is the quantity of these growth-promoting substances.

These organic additions appear to have a most marked effect on the nuclei of the plants so treated, and it was thought to be probable that the active agent might be some essential constituent of the nucleus. Bottomley [1917, 2] has shown that raw peat contains nucleic acid derivatives, particularly a dinucleotide, and more recently [1919] he has also shown that these nucleic acid derivatives have a marked stimulating effect on plant growth. The bacterised peat contains a very small quantity of nucleic acid, or dinucleotide, if any at all, but a proportionately larger quantity of purine and pyrimidine bases, and so it appears probable that it is the derivatives of the nucleic acid, rather than the acid itself, which has the auximonic effect.

An examination was accordingly made of the various organic composts with which the above experiments were carried out, to ascertain whether they contained either nucleic acid or its constituent groupings, which, indi-

vidually or collectively, might function as growth-promoting substances. At the same time a typical sample of the *Sphagnum* which gives rise to the peat was also examined for the occurrence of these substances; and not only was the *Sphagnum* so examined, but also a comparison was made between a sample of peat from the very surface of the deposit, and a sample from a depth of about 12 feet, in order to ascertain the effect, if any, on the nucleic acid constituents, of the changes which had resulted in the conversion of the *Sphagnum* into peat.

Extraction Experiments.

Methods of extraction.

Various methods of isolation were tried. The usual method of extracting with caustic soda and acidifying the extract with hydrochloric acid to remove the humic acids always resulted in a loss of the desired substances, which were apparently adsorbed in the humic acid precipitate, for this always contained phosphoric acid and showed an abnormally high nitrogen content. Schreiner and Lathrop's modification [1912], by which the alkaline extract was acidified with acetic instead of hydrochloric acid, to avoid the precipitation of any of the nucleic acid, gave slightly better results; but the extraction with caustic soda was very unsatisfactory, on account of the difficulty of filtration with the large quantities of material employed. Where this method was employed at all, a further modification was introduced which obviated this difficulty to some extent. The material was well shaken with a 1 % solution of caustic soda in a shaking machine for several hours, to ensure a thorough extraction, and the material was then, before filtration, acidified with the required acid (hydrochloric or acetic or both). This rendered filtration much more easy, and obviated the necessity of further precipitating and removing the humic acids. Whenever caustic soda was used, this method was followed out.

The use of sodium carbonate as the extractive was an improvement upon the caustic soda, for the mixture filtered much more readily, but in any case the extraction of the humic acid with the nucleic acid was very inconvenient, and it became desirable, if possible, to employ a method which avoided the solution of the humic acid.

The employment of sodium chloride as the extractive, according to the method of Clarke and Schryver [1917], was also tested upon all the materials examined, but the amount of material extracted in this way was much smaller than that obtained by the alkaline extraction, and this method was therefore discontinued.

After much experiment sodium bicarbonate was finally found to be the best extractive to employ for the separation of the nucleic acid constituents, for this reagent separates as large a quantity of the required substances as does the caustic soda, without dissolving the humic acids at all, so that the trouble of removing the latter, and the loss of nitrogen bases by adsorption

in the precipitate, are avoided. The method generally employed for extracting all of the composts investigated was therefore as follows. The material was air-dried and ground as finely as possible. A weighed quantity, which varied with the density of the material used, was put into a three-litre bottle, and generally sufficient was used to about half-fill the bottle, which was then almost filled with a 1 % solution of sodium bicarbonate. The bottle was tightly corked, and shaken on a shaking machine for several (four to six) hours. The contents were then filtered through paper in a Buchner funnel by the aid of a pump, and the residue again extracted with a fresh quantity of bicarbonate solution. This was repeated a third, and sometimes a fourth, time, if the third extraction showed much coloration. A fresh weighed quantity of the material was similarly treated, and this was repeated until from five to ten kilos of the compost had been extracted. The combined extracts were acidified with acetic acid, to precipitate any protein, and the slight precipitate was allowed to settle. The supernatant liquid was decanted off, just neutralised with sodium carbonate solution, and concentrated *in vacuo* to a comparatively small volume. The liquid was then again filtered by the aid of a pump, and poured into a mixture of about five times its own volume of absolute alcohol acidified with hydrochloric acid. A flocculent precipitate appeared which was more or less copious depending on the compost under examination, and this was allowed to settle for about 24 hours. The supernatant liquid was then decanted off through a filter, and more absolute alcohol added to the precipitate, which was well stirred and allowed to settle once more. The alcohol was again decanted off through a filter, and this washing was repeated once more. The precipitate was then dried in a vacuum desiccator, and the filtrate and washings were added together and preserved for examination.

This precipitate was white in appearance, or, in some cases, slightly yellowish. From the method of its preparation, and its appearance, the substance was presumed to be nucleic acid, or a derivative of it, and in each case it was examined for the typical constituent groupings of this acid. For the sake of convenience it will be referred to as the "nucleic acid precipitate."

A. *Examination of the "Nucleic Acid Precipitate."*

The material was first tested for phosphoric acid by incinerating a small portion and applying the ammonium molybdate test to the ash. The powder was then subjected in each case to two methods of hydrolysis, (*a*) a method of mild hydrolysis, in which the substance was heated at 100° with 10 % sulphuric acid under a reflux condenser for about two or three hours; and (*b*) a method of stronger hydrolysis, in which sulphuric acid was used at a temperature of 140° for about six hours. In all cases this second method was found to be more effective. Both methods always resulted in the production of a brown deposit of humic acids, indicating the presence of a carbohydrate radicle.

Separation of Purine and Pyrimidine Derivatives (I).

Two methods were then employed for the separation of purine and pyrimidine derivatives, these methods being slight modifications of those recommended by Jones [1914]. The first method involved the use of a silver salt. The hot liquid, after hydrolysis, was diluted with hot distilled water and filtered to remove the deposit of humic acids. A hot saturated solution of barium hydroxide was added until all the sulphuric and phosphoric acids had been precipitated, together with some of the colouring matter, and the liquid was alkaline to litmus. After removal of the precipitate by filtration, the excess of baryta was removed by carbon dioxide, and the pale straw-coloured liquid was concentrated *in vacuo* to a comparatively small volume. A little nitric acid was then added, followed by a saturated solution of silver nitrate, so long as a precipitate formed. This precipitate of silver-purine was filtered off, washed on the filter with very dilute nitric acid, and further examined as described below.

The filtrate and washings were thoroughly cooled, and a solution of silver nitrate further added until the addition of a drop of cold barium hydroxide solution resulted in the production of a yellow precipitate. The liquid was then made slightly alkaline to litmus with a cold saturated solution of barium hydroxide, and the silver-pyrimidine precipitate filtered off for examination.

(*a*) *Examination of the Silver-Purine Precipitate.*

The silver-purine precipitate was suspended in hot water, decomposed with dilute hydrochloric acid, and the precipitate of silver chloride removed. The liquid was then made strongly alkaline with ammonia at the boiling point, boiled briskly for a few minutes to precipitate guanine, and filtered, the precipitate being washed with dilute ammonia. The filtrate and washings were again brought to the boiling point, and a hot saturated solution of picric acid added. This precipitated adenine in the form of clusters of small needle crystals, which could be separated by filtration.

The guanine and the adenine picrate, where they occurred, were purified and used for the preparation of other salts, by which their identity was confirmed.

(*b*) *Examination of the Silver-Pyrimidine Precipitate.*

The precipitate of silver-pyrimidine was suspended in hot water and decomposed with sulphuretted hydrogen, the precipitate of silver sulphide being removed by filtration. A little barium in the filtrate was removed by sulphuric acid, and the liquid concentrated *in vacuo*. A hot saturated solution of picric acid was added in order to precipitate any cytosine present as the crystalline picrate. After standing for 24 hours, the cytosine picrate was filtered off, and purified by recrystallisation from hot water.

The filtrate was shaken with sulphuric acid and ether in order to remove

the picric acid, and the sulphuric acid was in turn removed with baryta. After concentration, the filtrate was allowed to stand, in order to allow of the separation of any uracil present.

Separation of Purine and Pyrimidine Derivatives (II).

The second method employed for the examination of the hydrolysed product concerned chiefly the purine bases. The hot liquid was diluted and filtered free of humic acids as before, and then concentrated ammonia was added at the boiling point until the guanine was completely precipitated together with the phosphate. The whole was boiled briskly for a few minutes and the precipitate then filtered off and washed with dilute ammonia. The precipitate was freed from phosphate by treating it with warm dilute caustic soda, which dissolved the guanine while the phosphate remained insoluble. The liquid was then filtered free from the latter, and the guanine precipitated from solution by means of dilute acetic acid, and used for the preparation of various salts for purposes of identification.

The filtrate and washings from the ammoniacal precipitate were added together and acidified with sulphuric acid. From this liquid the adenine was precipitated as the copper compound in the usual way by boiling the liquid, to which copper sulphate had been added, with a saturated solution of sodium bisulphite, which was added until the precipitate formed began to show a yellowish appearance. The whole was then boiled rapidly for a few minutes, and the precipitate of copper-purine filtered off, washed well with boiling water, suspended in hot water and decomposed with sulphuretted hydrogen. The filtrate from the copper sulphide was concentrated and used for the preparation of various salts of adenine.

The filtrate from the copper-purine compound, after removal of the copper with sulphuretted hydrogen and of the sulphuric acid with baryta, was either treated with silver nitrate and baryta for the precipitation of pyrimidine derivatives as above, or these bases were separated by the use of a mercuric salt. A solution of mercuric sulphate was added to the liquid, which was acidified with sulphuric acid, and the whole allowed to stand for 24 hours. The precipitate was filtered off, decomposed with sulphuretted hydrogen, and the process continued as in the examination of the pyrimidine-silver precipitate after removal of the silver.

(*c*) *Examination for Presence of Carbohydrate Radicle.*

The so-called "nucleic acid precipitate" was in every case tested by Molisch's reaction for carbohydrates. After mild acid hydrolysis, and also after hydrolysis by ammonia, the products were tested for reducing sugars by Fehling's solution, and for pentoses by the phloroglucinol and orcinol tests.

B. *Examination of the Filtrate after Removal of the "Nucleic Acid Precipitate."*

The filtrate, after removal of the "nucleic acid precipitate," was just neutralised with ammonia, and the alcohol distilled off *in vacuo*. The aqueous fluid was tested for the presence of phosphoric acid by both magnesia mixture and ammonium molybdate. The presence of carbohydrates was also investigated by means of the tests described above.

A preliminary examination of the remainder of the filtrate showed that in order to separate the bases, acid hydrolysis was unnecessary, for they were readily separated from the fluid by the employment of the silver, copper and mercuric salts. All of these materials were used for the further examination of the filtrate for purine and pyrimidine substances, the methods employed being those already described.

Results obtained with the Different Organic Materials.

(*a*) *Sphagnum Moss.* A fairly copious precipitate of so-called "nucleic acid" was obtained by both the caustic soda and the sodium bicarbonate extraction. This precipitate gave a good reaction for phosphoric acid when incinerated, and gave positive results with Molisch's test for carbohydrates. After both mild acid and alkaline hydrolysis, the products reduced Fehling's solution and responded to the orcinol and phloroglucinol tests for pentoses.

Both of the purine bases which occur in yeast nucleic acid, adenine and guanine, were isolated from the hydrolysed material, the former in greater proportion than the latter. Adenine was recognised by (1) the characteristic form of its picrate, which occurred in clusters of fine needles, giving on recrystallisation elongated prisms which melted with decomposition at about 275°; (2) the hydrochloride, occurring as flat deliquescent prisms; (3) the dichromate, as six-sided plates; (4) the double salt with gold chloride, which appeared as elongated orange-coloured prisms. This base also responded to Kossel's test for purines, gave a gelatinous precipitate with ammoniacal silver nitrate, and a red colour, unchanged by heating, with ferric chloride.

Guanine was recognised by the formation of (1) the hydrochloride, as long needle crystals; (2) the dichromate, forming orange coloured prisms with truncated ends; (3) the picrate, a woolly mass of long thread-like needles when first formed, drying to a compact felt-like mass which became orange-red on heating, and decomposed without melting at about 190°; (4) the sulphate, forming needle crystals which separated out rapidly on cooling a hot solution in sulphuric acid. The solubility of this substance in hydrochloric acid and in ammonia was also characteristic; besides which, a small portion evaporated with a drop of concentrated nitric acid on porcelain left a muddy yellow spot which gave a brownish-red colour with sodium hydroxide, indicating the presence of guanine.

On examining for pyrimidine bases, both cytosine and uracil were isolated, the latter being greater in quantity than the former. Cytosine was recognised by the formation of (1) the hydrochloride, in the form of needle crystals, (2) the picrate, as almost insoluble needles, (3) the chloroplatinate, in the form of six-sided plates. The free base was also obtained in the form of plates by treating the solution of the chloride with ammonia, and this gave the colour reaction of Wheeler and Johnson, which is characteristic of pyrimidines.

The uracil was identified by its method of preparation, by its failure to form an insoluble picrate, and by the clusters of needle crystals formed from a sulphuric acid solution, which gave the colour reaction of Wheeler and Johnson.

The filtrate from this "nucleic acid precipitate" also gave the well-marked reactions for phosphoric acid and for carbohydrates (pentoses), which, of course, would not necessarily arise from nuclear material; besides which each of the purine and pyrimidine substances, adenine, guanine, cytosine and uracil, was present in fair quantity, and was recognised in each case by the preparation of the salts and the application of the tests mentioned above.

The *Sphagnum* moss, therefore, evidently contains some true nucleic acid, which is precipitated by acid alcohol from an alkaline extract; but in view of the larger proportion in which both adenine and uracil occur, as compared with guanine and cytosine, in the hydrolysed material, it is evident that the precipitate does not consist wholly of nucleic acid, but contains also an admixture of the adenine-uracil dinucleotide which occurs in peat [Bottomley, 1917, 2]. Besides these, the *Sphagnum* also contains the free purine and pyrimidine bases from nucleic acid; so that, broadly, it may be said that the nucleic acid constituents of *Sphagnum* consist of some true nucleic acid, a smaller quantity of adenine-uracil dinucleotide, and some free purine and pyrimidine derivatives resulting from the disintegration of the nucleic acid.

(*b*) *Surface Peat.* A peat taken from the very surface of the deposit, and still retaining something of the colour of the original *Sphagnum*, was similarly investigated. This yielded a smaller quantity of "nucleic acid" precipitate than did the *Sphagnum*, but on hydrolysis the material showed relatively much more adenine and uracil, and less guanine and cytosine than did the *Sphagnum* material. Carbohydrate (pentose) and phosphoric acid were also present in the products of hydrolysis. Evidently, therefore, it contained a smaller proportion of nucleic acid as compared with adenine-uracil dinucleotide than did the former.

The filtrate from the "nucleic acid precipitate" also contained carbohydrate, phosphoric acid, and both purine and pyrimidine bases, viz. adenine, guanine, cytosine and uracil, and these occurred in relatively larger proportion, as compared with the "nucleic acid precipitate," than in the *Sphagnum* moss. The purine and the pyrimidine substances were identified by the same products and tests as indicated above.

These results indicate that in the surface peat disintegration of the original

nucleic acid has already taken place to a certain extent, the products being a larger proportion of dinucleotide and free bases as compared with the original *Sphagnum*.

(*c*) *Deep Peat.* A peat taken from about 12 feet below the surface of the deposit was investigated, and a certain quantity, which was less than in either of the other cases, of "nucleic acid precipitate" obtained. On hydrolysis, this was found to contain carbohydrates and phosphoric acid, and of the nitrogen bases adenine and uracil, but no guanine or cytosine. It therefore contained no true nucleic acid at all, but consisted entirely of adenine-uracil dinucleotide. This is in agreement with the results of Bottomley.

The filtrate from this precipitate contained also carbohydrate and phosphoric acid, and a relatively large quantity of nitrogen bases. Adenine, guanine, cytosine and uracil were all isolated and identified as above, but the purine bases occurred in much larger quantity than the pyrimidines. This may be due to loss by leaching during the long period of the peat formation, or it may be due to chemical or bacterial changes during the decay of the plant substance, which changes have attacked the pyrimidine substances to a greater extent than the purines.

During the process of peat formation, it is evident that the original nucleic acid has undergone extensive disintegration, probably first of all into dinucleotides, of which a comparatively small quantity remain, and finally into the free constituent bases, which therefore occur in relatively larger quantity in the deeper peat than in either the original *Sphagnum* or the surface peat, but of which a proportion of the pyrimidine bases has disappeared.

(*d*) *Bacterised Peat.* An examination of bacterised peat was made by both the caustic soda and the sodium bicarbonate methods. Only a very small quantity of "nucleic acid precipitate" was obtained, but this was found to be in all probability a true nucleic acid, yielding all four nitrogen bases in approximately equal proportion. In view of the absence of nucleic acid from the deep raw peat which is used in manufacture, this small quantity is evidently a synthetic product formed during bacterisation, though at present its origin is obscure.

The filtrate from this deposit contained a fair quantity of all of the four nitrogen bases, though here again the adenine and guanine far exceeded in quantity the cytosine and uracil, as in the case of raw peat.

Thus it appears that the further bacterial action has resulted in the breaking down of the adenine-uracil dinucleotide into its constituent groupings, and these are very largely present in a water extract of the material, which does not contain any of the nucleic acid extracted by alkalies.

(*e*) *Leaf-mould.* A sample of good and fairly pure leaf-mould was extracted by caustic soda, sodium carbonate and sodium bicarbonate, and all three methods yielded similar results. A small quantity of material was obtained by the acid-alcohol precipitation, and this yielded on hydrolysis, adenine, guanine, cytosine and uracil, besides carbohydrates and phosphoric

acid. The guanine and cytosine, however, were present only in very small quantity as compared with the adenine and uracil, so that the material evidently contained a comparatively small proportion of true nucleic acid, the remainder being presumably adenine-uracil dinucleotide. The filtrate from this "nucleic acid precipitate" contained all four nitrogen bases in relative abundance, besides more carbohydrate and phosphoric acid. The leaf-mould, therefore, contains a fair quantity of nucleic acid derivatives, by far the greater proportion being in the form of the free bases, while only a small fraction exists as true nucleic acid, with a larger quantity of dinucleotide. In the sample investigated the disintegration of the original nucleic acid into its constituent groupings had apparently been almost complete.

(*f*) *Fresh Stable Manure.* This material was extracted by both caustic soda and sodium bicarbonate, and in each case a small quantity of the precipitate with acid-alcohol was obtained. This material consisted almost entirely of nucleic acid, for it gave on hydrolysis adenine, guanine, cytosine and uracil in practically equal proportions, so far as could be judged. The filtrate contained also comparatively large amounts of all these four bases, together with small quantities of xanthine and hypoxanthine.

The nucleic acid in this compost had probably been derived from the plant material constituting the animal's food, and the greater proportion had evidently been decomposed into the free bases in its passage through the animal's body. Since the animals are practically entirely herbivorous, the whole of these nucleic acid derivatives evidently owe their origin ultimately to the elements contained in their plant food. But the purine and pyrimidine bases have by no means necessarily been derived wholly, or even very largely, directly from the plant nucleic acid. On the contrary, a large proportion may be presumed to have arisen as products of metabolism in the animal's body, being elaborated from the plant materials supplied.

(*g*) *Well-rotted Stable Manure.* This was extracted in just the same way as for the fresh manure, and only a very slight "nucleic acid precipitate" was obtained. On hydrolysis this material yielded adenine and uracil as the only nitrogen bases, so that evidently no true nucleic acid was present, the precipitate being dinucleotide.

In the filtrate from this material were found all the free bases as indicated above for the fresh manure, but they occurred in rather larger quantity. It was noticeable here that the adenine and guanine were relatively the most abundant, occurring in greater quantity than the pyrimidine derivatives, which had evidently either been leached out, or had been transformed by the action of enzymes or micro-organisms. The filtrate also showed the presence of carbohydrate and phosphoric acid. The alkaline extract was much darker in colour, giving a much more copious precipitate of humic acids on acidification, which had, no doubt, arisen by bacterial action on the cellulose and soluble carbohydrates in the animal's food.

In the rotted stable manure, therefore, the decomposition of the nucleic

acid has proceeded to such an extent as to resolve it completely into its constituent bases.

(*h*) *Soil.* A sample of the same good fertile soil as that which was used in the *Lemna* experiments, and which had been well-manured for several years, was also extracted with caustic soda, sodium carbonate and sodium bicarbonate. A small quantity of the "nucleic acid precipitate" was obtained by each method, and on hydrolysis yielded in each case adenine, guanine, cytosine and uracil, the first and last being in greater proportion than the other two. Phosphoric acid and carbohydrate were also found amongst the products of hydrolysis, so that the precipitate evidently contained some nucleic acid together with adenine-uracil dinucleotide.

From the filtrate from this precipitate were isolated all of the four nitrogen bases from plant nucleic acid, viz. adenine, guanine, cytosine and uracil, together with xanthine and hypoxanthine and a little carbohydrate. All of these substances, however, occurred in relatively much smaller quantity than in any of the other materials examined, while as might have been expected, phosphoric acid occurred in much greater quantity. Among the purine bases, adenine was found to occur in greater quantity, and all of the purine bases were present in greater quantity than the pyrimidines, of which the proportions were extremely small. The occurrence of xanthine, hypoxanthine and adenine among purine bases, has already been reported in soil [Schreiner and Shorey, 1910; Shorey, 1913] and of the pyrimidine derivatives cytosine has also been identified [Schreiner and Shorey, 1910]. The soil under examination here showed the presence of all four of the purine bases mentioned, as well as both of the pyrimidine derivatives, in varying quantities, but it is not to be expected that all soils will necessarily show the presence of the same substances, nor that the composition of any one soil will remain constant for any length of time, in view of the multiplicity of the bacterial and enzymic changes which are continually taking place.

The nucleic acid derivatives which have been found in the various composts examined can be briefly summarised in the following table:

Compost	Nucleic Acid	Dinucleotide	Free Nitrogen Bases
Sphagnum	Fair amount	Small quantity	Fair quantity of adenine, guanine, cytosine and uracil
Surface peat	Less than above	More than above	More than above
Deep peat	None	Less than surface peat	More than surface peat
Bacterised peat	Trace	None	Still more than above
Leaf-mould	Very little	Small quantity	Fairly large amounts of four bases as above
Fresh stable manure	Small quantity	Practically none	Fair amount of adenine, guanine, cytosine and uracil; small amounts of xanthine and hypoxanthine
Well-rotted stable manure	Practically none	Very small quantity	Rather more adenine and guanine than above; less cytosine and uracil; small amounts of xanthine and hypoxanthine
Soil	Small quantity	Small quantity	Small amount of adenine; less guanine, xanthine and hypoxanthine; still less cytosine and uracil

Summary and Conclusion.

The ordinary organic manures which are generally applied to soils in agricultural and horticultural operations, viz. leaf-mould, fresh and well-rotted stable manures, have been shown to contain, in varying proportions, water-soluble substances which are effective as plant growth-promoting substances, or auximones. Such ingredients are also to be found in well-manured fertile soil, for water extracts of all these materials, when added in certain proportions to inorganic nutrient solutions, promote the healthy growth and development of plants of *Lemna major*, which without such additions rapidly become unhealthy and diminish in weight.

A comparison between fresh and well-rotted stable manure shows that in whatever proportions the materials are used, in all cases the extract of rotted manure is more effective than that of the fresh material, while an extract of peat which has been artificially subjected to bacterial decomposition under controlled conditions is more effective than either. The increase in growth following upon the addition of these materials to the culture solution is such as can not be attributed to the purely nutritive value of the materials added, and all these composts therefore must be assumed to contain growth-promoting substances, or auximones. The amount of these substances varies directly with the extent of the bacterial decomposition of the material, which decomposition is an important factor in determining the efficacy of the compost from a practical standpoint.

The exact nature of the essential substances is still very problematical, but Bottomley [1917, 2] has shown that peat contains derivatives of nucleic acid, and [1919] that these derivatives are effective in increasing the growth of plants when added to culture solutions. Microscopic examination has shown that the various auximones have a marked influence on the size and the contents of the cell, especially on the nucleus, and it seemed not improbable that they might supply some essential nuclear constituent. A detailed examination was therefore made of all of the above materials, viz. well-manured soil, leaf-mould, fresh and well-rotted stable manures, to determine whether they contain derivatives of nucleic acid; and at the same time an examination was made of *Sphagnum* in various stages of its conversion into decomposed peat, in order to ascertain what changes, if any, these materials have undergone during such conversion.

As a result it has been found that all these materials contain appreciable quantities of nucleic acid and its derivatives in various stages of decomposition. Generally speaking, nucleic acid, a dinucleotide, and the free purine and pyrimidine bases have all been found to occur in varying proportions, and the greater the bacterial decomposition of the material concerned, the greater is the resolution of the original nucleic acid into its free bases.

The greater the extent of the decomposition, the more effective is a water extract of the material when added to a culture solution, and the greater is the value of the material from a practical point of view. It would

therefore appear, assuming that the whole effect is due to nucleic acid or its derivatives, that the free bases, individually or collectively, are of more value as growth-promoting substances than the nucleic acid. Schreiner, Reed and Skinner [1907] have found that xanthine and guanine, in concentrations of 40 parts per million or less, increased the growth of wheat seedlings in water culture. Their experiments were made on ordinary seedlings with a fairly large reserve of organic matter in their endosperm, and were of only 10 to 12 days' duration, while in all the experiments carried out by Bottomley and the writer, it has been found that the beneficial effect of the added organic substance is best manifested after 21 days from the commencement of the experiments, when the original supply of auximones in the plant has presumably become either exhausted or so diffuse as to be insufficient for the needs of the plant. Similarly, Schreiner and Skinner [1912] have shown that nucleic acid, hypoxanthine, xanthine and guanine are beneficial under similar conditions, the guanine especially to root development, and a noticeable feature of the experiments recorded above was the marked improvement in the health of the roots. If the experiments of these authors had been carried out over longer periods and with plants which have not a large organic reserve to start with, it is most probable that the added organic materials would have proved even more beneficial. The effect, however, was quite marked, xanthine for instance giving an increase of 21 % in the gross weight of the plants. It is proposed to continue the present work by investigating the action of the free bases, separately and collectively, on the growth of plants which reproduce themselves in a similar manner to that of the *Lemnaceae*.

The value of organic manures is a well-recognised fact, and stress has been laid on their physical effect on the texture of the soil, and on the value of the organic matter as food for soil bacteria, but the fact that the organic materials may be of direct importance to the plant has received very little attention. Livingston [1907] acknowledged this fact when experimenting on the effect on plant growth of extracts of green and stable manures. He added these materials to a slightly modified Knop's solution, and found that "by far the most important factor in the effectiveness was the organic matter," which he found to be of direct value to the plant, although in what lay its effectiveness was not clear. In the experiments recorded here it has been shown that all of the organic manurial composts are of direct value to the plant, and that they all contain nucleic acid or its derivatives, which have been shown by Bottomley and the authors quoted above to be beneficial to plant growth, serving as growth-promoting substances, or auximones. It is evident, therefore, that part, at least, of the beneficial effect of organic manures must be due to the direct value of these substances to the plants. It is not suggested that these nucleic acid derivatives are the only auximones present in the organic composts. In such accumulations of material, the composition of which never remains constant, but is continually undergoing change on account of bacterial

and enzyme action, it is quite probable that there are many such substances capable of functioning as auximones, but the nucleic acid derivatives certainly play an important part. These auximone substances probably occur partly as degradation products of the organic debris, partly as direct products of the synthesis of moulds and bacteria, for it has been shown that auximones do occur as synthetic products of yeast [Williams, 1919, and Bachmann, 1919], and of nitrogen-fixing bacteria [Bottomley, 1919]. Whatever their origin, their liberation in the soil organic matter is evidently due to the action of micro-organisms. These organisms play a part in the auximone cycle not inferior to that of the green plant, which may be regarded as acting as a carrier, absorbing the auximones, which are liberated by the soil organisms, and utilising them in its own metabolism, in the course of which it elaborates the vitamines which in their turn are passed on to the animals that require them.

In conclusion, I wish to express my gratitude to Professor W. B. Bottomley for supervising this work, and to the Department of Scientific and Industrial Research for a grant which enabled it to be carried out.

REFERENCES.

Bachmann (1919). *Journ. Biol. Chem.*, **39**, 235.
Bottomley (1914). *Ann. Bot.*, **28**, 531.
—— (1917, 1). *Proc. Roy. Soc.* B, **89**, 481.
—— (1917, 2). *Proc. Roy. Soc.* B, **90**, 39.
—— (1919). *Proc. Roy. Soc.* B, **91**, 83.
—— (1920, 1). *Ann. Bot.*, **34**.
—— (1920, 2). *Ann. Bot.*, **34**.
Clarke and Schryver (1917). *Biochem. J.*, **11**, 319.
Jones (1914). *Nucleic Acids.* (Longmans.)
Livingston (1907). U.S. Dept. Agric., Bureau of Soils, *Bull.*, 36.
Mockeridge (1917). *Proc. Roy. Soc.* B., **89**, 508.
Schreiner and Lathrop (1912). U.S. Dept. Agric., Bureau of Soils, *Bull.* 89.
—— Reed and Skinner (1907). U.S. Dept. Agric., Bureau of Soils, *Bull.* 47.
—— and Shorey (1910). U.S. Dept. Agric., Bureau of Soils, *Bull.* 74.
—— and Skinner (1912). U.S. Dept. Agric., Bureau of Soils, *Bull.* 87.
Shorey (1913). U.S. Dept. Agric., Bureau of Soils, *Bull.* 88.
Williams (1919). *Journ. Biol. Chem.*, **38**, 465.

XXXVIII. RAPID VOLUMETRIC METHODS FOR THE ESTIMATION OF AMINO-ACIDS, ORGANIC ACIDS AND ORGANIC BASES.

By FREDERICK WILLIAM FOREMAN.

From the Institute for the Study of Animal Nutrition, School of Agriculture, Cambridge University.

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In the course of an investigation of the process of putrefaction, it became necessary to devise methods by which many estimations could be made of such products of enzyme or bacterial action as organic acids, amino-acids and organic bases. Existing methods such for instance as that for determining the organic acids volatile in steam, the estimation of amino-acids by Sörensen's formol titration method and the separation of organic bases by distillation were tried but found wanting either on account of the lengthy nature of the process or the inaccuracy of the results.

It was recognised that volumetric methods which would make possible the rapid and accurate estimation of mixtures of organic acids, amino-acids and organic bases would have such wide applications that it would be worth while devoting much time and trouble to the subject. The investigation has resulted in the discovery of a simple and accurate volumetric method which admits of general application to the analysis of such organic mixtures as result from the degradation of proteins or similar bodies by acids, enzymes or bacteria.

On treating a fluid in which putrefaction has been proceeding with nine volumes of alcohol as described in a previous paper [Foreman and Graham-Smith, 1917] proteins, albumoses, peptones and other substances whose presence might interfere with the estimation of simpler constituents are precipitated. The filtrate which is light yellowish brown in colour contains about 87 % alcohol. The clue which led up to the evolution of a successful method applicable to such alcoholic filtrates was found in an observation by Sutton [1900] that potash or soda will displace ammonia in equivalent quantities from ammonium salts in alcoholic solution at ordinary temperatures, whilst at the same time the ammonia forms no compound with phenolphthalein used as indicator. The acid in an ammonium salt can therefore be titrated with standard alkali in alcoholic solution. Sutton does not state the strength of alcohol necessary to secure this result. The alcoholic filtrates contain amines as well as ammonia together with about 13 % of water. It seemed reasonable

to assume that the amines might behave as ammonia towards phenolphthalein in alcoholic solution, in which case the bases would not interfere with the direct quantitative titration of the organic acids in the alcoholic filtrates provided the presence of a little water did not vitiate the results.

In order to provide a stock of standard material on which the method could be tested, the following procedure was adopted.

The body of a rabbit, including the skin and internal organs with their contents, was minced, and $2\frac{1}{2}$ times the weight of water added in a Winchester quart bottle. The bottle was loosely plugged with cotton wool and kept at 25–30° for four weeks, in an inclined position so as to expose as great a surface as possible. The contents were lightly shaken once daily, the plug being removed during this operation. At the end of the period the fluid contained the products of advanced aerobic putrefaction. A portion was treated with nine volumes of 97 % alcohol, allowed to stand all night and filtered. The solution containing 87·3 % alcohol was stored in a stoppered bottle and will be referred to frequently in this paper as the "Alcoholic Extract." The following results were obtained on analysis of 50 cc. portions by the methods described in an earlier communication [Foreman and Graham-Smith, 1917].

Volatile bases	24·85 cc. N/10 acid
Formol titration of residual liquid (amino-acids)	2·1 cc. N/10 soda
Organic acids volatile in steam	19·7 cc. N/10 soda

The "Alcoholic Extract" was titrated under the differing conditions described in Table I, using phenolphthalein as indicator. The 97 % alcohol used was slightly acid to phenolphthalein, 100 cc. requiring 0·15 cc. N/10 alkali.

Table I. Preliminary Experiments with the "Alcoholic Extract."

Col. 1	2	3	4	5
Ref. No.	Reagents added to 10 cc. portions of "Alcoholic Extract" before titrating	Aq. N/10 soda required cc.	% alcohol in the mixture after titrating	Readings calculated to a basis of 50 cc. "Extract," after correcting for the original acidity of the alcohol
1	0	4·2	61	20·1
2	20 cc. 97 % alcohol	4·7	81	23·35
3	50 cc. „	4·7	88·5	23·13
4	50 cc. 97 % alcohol and 0·5 cc. ammonia (sp. gr. ·88)	4·7	87·8	23·13
5	50 cc. 97 % alcohol and 5 cc. aq. butyric acid solution (=7·6 cc. N/10 soda to phth.)	12·25 7·6 4·65	73·9	22·88
6	100 cc. 97 % alcohol and 5 cc. aq. butyric acid solution	12·4 7·6 4·8	83·0	23·25
7	50 cc. 97 % alcohol	4·75	88·5	23·38
8	To the titrated liquid of No. 7 added a neutralised mixture of 10 cc. formalin and 50 cc. 97 % alcohol	up to 4·85	84·7	23·88

The following observations were made during the carrying out of the first six experiments described in Table I. Reference will be made later to Exps. Nos. 7 and 8.

1. The light yellowish brown colour of the "Alcoholic Extract" practically disappeared on diluting with alcohol.

2. The endpoints were quite satisfactory.

3. On raising the percentage of alcohol from 61 to 81 the reading was increased, but between 81 and 88·5 % no further increase occurred.

4. The result was greater than previously obtained for the volatile organic acids in the same quantity of "Alcoholic Extract."

5. The presence of even a large amount of free ammonia did not affect the reading although the mixture contained 12 % of water.

6. The butyric acid added titrated quantitatively in the mixture.

The results showed that ammonia liberated by the alkali from the ammonium salts, which the "Alcoholic Extract" contained, did not affect the indicator when 81 to 88 % of alcohol was present. This inference was confirmed by obtaining quantitative results for the acid radicle on titrating a known quantity of pure ammonium chloride in 85 % alcohol.

As the "Alcoholic Extract" contained amines as well as ammonia, the behaviour of primary, secondary and tertiary amines towards phenolphthalein in alcoholic solutions was investigated. Known quantities of the pure hydrochlorides of these bases were titrated in 85 % alcoholic solution and found to require $N/10$ alkali quantitatively equivalent to the acid radicle in each case. The actual experiments will be found in a later section. These amines therefore resembled ammonia in forming no compound with phenolphthalein in the alcoholic solutions.

The effect of alcohol in different concentrations upon the titration value of the acid radicles when titrated in the presence of the free bases was then studied in detail. For this purpose 97 % alcohol was added in small quantities at a time to known quantities of standard aqueous solutions of ammonium salts and salts of primary, secondary and tertiary amines, and the liquids titrated with $N/10$ alkali after each addition using phenolphthalein as indicator. The total titration value increased as the content of alcohol in the solutions was augmented, reaching a maximum when 80 to 85 % alcohol was present. In the experimental section, a typical experiment of this nature carried out with trimethylamine hydrochloride is fully described. In this case the reading reached its highest point when the concentration of alcohol had been increased to 78 %.

Having determined that ammonia and amines form no compound with phenolphthalein in the presence of 85 % alcohol, the amount of $N/10$ alkali required to neutralise a certain volume of the "Alcoholic Extract" under this condition was regarded, at this stage of the investigation, as quantitatively equivalent to the organic acid radicles present. An attempt was therefore made to obtain a direct figure for the amino-acids in the "Alcoholic

Extract" by continuing the titration after adding a neutral alcoholic solution of formalin to the neutralised alcoholic solution. Experiments Nos. 7 and 8 of Table I, were therefore carried out. The additional $N/10$ soda required for neutralisation on adding the alcoholic formalin, however, was only 0·5 cc. per 50 cc. "Alcoholic Extract," whereas a reading of 2·1 cc. $N/10$ soda for the amino-acids in the same volume of "Alcoholic Extract" had been obtained previously by the formol titration of the residual liquid from the distillation of the volatile bases (p. 471). In attempting to find a reason for this deficiency, the behaviour of amino-acids was investigated under the same conditions. A 0·53 % aqueous solution of an amino-acid mixture, consisting of leucine and valine and containing 11·34 % N, was therefore treated and titrated as described in Table II.

Table II. Preliminary experiments with amino-acids.

Col. 1	2	3	4
Ref. No.	Treatment of the 0·53 % aq. solution of amino-acids before titrating	Aq. $N/10$ soda required (corrected) cc.	% alcohol in the mixture after titrating
9 (*a*)	5 cc. + 50 cc. 97 % alcohol	1·97	85·0
(*b*)	5 cc. + 100 cc. „	1·97	90·7
10	To the titrated liquid of Exp. 9 (*b*) added a neutralised mixture of 10 cc. formalin and 50 cc. 97 % alcohol	2·17	87·0

The result of Exp. No. 9 of the above table was most astonishing. Assuming each amino-acid to require one equivalent of $N/10$ soda for neutralisation, 5 cc. of the leucine-valine solution would theoretically require, calculated on the N content, 2·15 cc. $N/10$ soda. The figure actually obtained was 1·97 cc., which remained constant on increasing further the concentration of alcohol. These two amino-acids therefore titrated almost quantitatively as ordinary monobasic organic acids in 85 % alcohol using phenolphthalein as indicator, although in aqueous solution they are practically neutral. The result also showed that the reading obtained when the "Alcoholic Extract" was titrated in the presence of 81–88 % alcohol (see Exps. Nos. 2 and 3, Table I) represented amino-acids and organic acids together, and not the organic acids alone, as originally supposed.

The amino-acid solution resembled the "Alcoholic Extract" in requiring a small additional amount of $N/10$ alkali on adding alcoholic formalin to the neutralised alcoholic liquids.

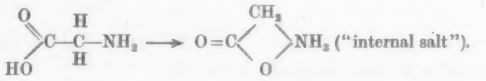
The surprising discovery that leucine and valine titrated in 85 % alcohol as if they were ordinary organic acids led to the supposition that this behaviour might prove common to amino-acids in general. If so, then the property might be utilised as a basis of a new titration method for the estimation of amino-acids. Other amino-acids were therefore submitted to investigation under the same conditions. The results of the experiments will be

given later. For convenience in presentation they may be summarised briefly as follows.

Phenylalanine, tyrosine, cystine, histidine, asparagine quantitatively required one equivalent of soda for neutralisation to phenolphthalein in the presence of 85 % alcohol, while the requirements of lysine and tryptophan were practically quantitative. Arginine was neutral. Glycine, alanine, leucine and valine gave a slight deficiency. The results obtained for aspartic and glutamic acid and proline however were considerably below the calculated value, with a corresponding lack of definiteness in the endpoints.

Hypothesis for explaining the behaviour of amino-acids in alcoholic solutions.

Assuming the neutrality of amino-acids in aqueous solutions to be due to the formation of "internal salts" as described by Mann [1906] an explanation of their behaviour in strong alcoholic solution on adding KOH seems possible. In the account given by Mann, glycocoll as a type is represented thus:



Walker [1904] believed that amino-acids in aqueous solution either form "internal salts" or unite in pairs in such a way that the basic radicle of one molecule links on to the acid radicle of another, the original trivalent N atom always becoming pentavalent whichever of these changes the amino-acid undergoes. Therefore the opinion was formed that the behaviour of amino-acids in alcoholic solutions on adding potash is comparable with that of an ammonium salt under the same conditions, the α-amino-group and ammonia being liberated from the "internal salt" and the ammonium salt respectively, and the potassium salts of the acid radicles formed. With its basic α-amino-group liberated from the "internal salt" the amino-acid can be regarded as a true substituted ammonia, which would be expected to behave similarly to ammonia and the simple amines in forming no compound with phenolphthalein in alcoholic solutions.

The effect of alcohol in different concentrations upon the basicity of the α-amino-group of amino-acids, was studied in the manner already described for investigating the basicity of ammonia and the amines (p. 453). The results obtained in two typical cases, viz.: histidine and arginine are given later (see Tables V and VI). Curves constructed from these data follow the same general contour as those obtained in a like manner for ammonium chloride and the hydrochlorides of the amines, the readings reaching the maximum at 80 to 85 % alcohol.

The abnormal behaviour shown by certain amino-acids titrated in alcoholic solution.

As already stated, certain amino-acids, more particularly aspartic acid, glutamic acid and proline, gave low results when titrated in their alcoholic solutions, although others behaved in accordance with the "internal salt" hypothesis (p. 455). Further work will be necessary before the deficiencies can be satisfactorily explained. Several possible explanations were considered. The following alternative suggestions both appear to receive support from the available evidence:

(1) That low titration values may be due to the union of a carboxyl group with alcohol.

(2) That condensation probably occurs in the alcoholic solutions.

At a much later stage, the discovery was made that the carboxyl groups of amino-acids will titrate in aqueous acetone in the same manner as they do in aqueous alcohol (see p. 457). Suggestion No. 1 is supported by the fact that aspartic acid, glutamic acid and proline, gave practically quantitative results when titrated in about 84 % acetone using phenolphthalein as indicator.

A comparison of the behaviour of aspartic acid and asparagine, on the other hand, seems to favour suggestion No. 2:

Aspartic acid	Asparagine
(*a*) $COOH$	$CONH_2$
$\vert$	$\vert$
CH_2	CH_2
$\vert$	$\vert$
$CHNH_2$	$CHNH_2$
$\vert$	$\vert$
(*b*) $COOH$	(*b*) $COOH$

Group (*a*) of aspartic acid titrated accurately in aqueous solution. On adding the stated amount of alcohol to this neutralised solution, group (*b*) required only about half the calculated equivalent of alkali to produce the faintest possible pink colour, the endpoint being very indefinite. The solution became faintly opalescent when the alcohol was added. These facts seem to support the suggestion that some kind of loose condensation, between group (*b*) of one aspartic molecule and the amino-group of another occurred in the alcoholic solution. Group (*b*) of asparagine however titrated quantitatively in alcoholic solution with a sharp endpoint. Therefore the phenomenon does not occur when no carboxyl group (*a*) is present. From this point of view it would appear that group (*a*) is involved in the condensation. Further investigation is proceeding.

Conditions under which the method becomes of general application.

The evolution of a successful general titration method for estimating amino-acids, based upon their behaviour in alcoholic solution, involved the necessity of overcoming the difficulty caused by certain amino-acids giving

low results. In endeavouring to achieve this purpose the significance of Exps. No. 8 of Table I and No. 10 of Table II was appreciated. Reference to these tables will show that the titration values of the "Alcoholic Extract" and the leucine-valine mixture, obtained in alcoholic solution, were slightly increased on adding alcoholic formalin, indicating that formaldehyde might be capable of disrupting any loose combination or condensation. A test was therefore made with glutamic acid. On adding alcoholic formalin to the neutralised alcoholic solution, sufficient further alkali was required to bring the total titration value up to the calculated figure. Subsequently every amino-acid giving a low result when titrated in alcohol alone was found to titrate quantitatively on adding alcoholic formalin. An accurate general method was therefore possible, provided those amino-acids which behaved normally in alcohol alone continued to do so in the presence of formaldehyde.

Histidine and tryptophan gave good results when titrated in alcohol alone. As these amino-acids contain an imino-grouping, methylene derivatives capable of neutralising acid when in aqueous solution are formed by the action of formaldehyde. Therefore it was necessary to show that such bases, which presumably would be formed on adding alcoholic formalin to the neutralised alcoholic solution of a complete amino-acid mixture, would be inactive to phenolphthalein, in which case they would not interfere in the general method. A study of the basicity of the basic methylene derivative of diethylamine, obtained by the action of formaldehyde upon the hydrochloride of this amine, showed that bases of this type, in common with ammonia, the amines and the freed amino-groups of amino-acids, do not form ionisable compounds with phenolphthalein if sufficient alcohol is present. The basic methylene derivative ceased to react with the indicator on increasing the concentration of alcohol to 70–75 %. The formation of basic methylene compounds of such amino-acids as histidine and tryptophan would therefore not be likely to vitiate the titration results. This statement was confirmed in actual experiments with these two amino-acids.

Low readings in alcohol raised on adding acetone.

Preliminary experiments have shown that acetone has the same effect as alcohol in submerging the basicity of the basic groups of amino-acids towards phenolphthalein. Aspartic acid, glutamic acid and proline, which gave low titration values in alcohol alone, titrated quantitatively in 80–85 % acetone. The behaviour of the other amino-acids has not yet been investigated. As an important differentiation of amino-acids in a mixture may be obtained by first titrating in 85 % alcohol and then continuing the titration after adding alcoholic formalin, the effect of adding acetone as an alternative to alcoholic formalin was tried in the case of aspartic and glutamic acids. These experiments, described later, showed that acetone was equally effective in raising the titration value to the quantitative figure. A thorough investigation of the effect of acetone is proceeding.

Description of the general method for estimating the carboxyl-groups of amino-acids.

Preparation of solution. A known weight of the amino-acid, or its suitable salt, or of an amino-acid mixture is dissolved in CO_2-free water and made up to a known volume so that the resulting solution is approximately $N/10$ in strength in relation to the carboxyl-groups. In cases such as tyrosine and cystine where the amino-acid is insoluble in water or soluble in too large a volume of water, $N/10$ HCl or $N/10$ soda or other appropriate means must be used for bringing them into solution so that no separation occurs on adding alcohol. When only a very small quantity of the amino-acid is available it may be weighed accurately into a suitable vessel dissolved in the desired amount of water and the whole of the solution used.

Stage 1. *Titration in water.* A 5 cc. or 10 cc. portion of the solution is titrated with aq. $N/10$ soda to phenolphthalein (soda standardised to phenolphthalein).

Stage 2. *Titration in alcohol.* 5 cc. or 10 cc. of the original solution is transferred to a conical flask of about 250 cc. capacity, 10 volumes of 97 % alcohol (rect. sp.) and three drops of phenolphthalein solution added, and the mixture titrated in daylight over a white plate with $N/10$ alcoholic potash (standardised to phenolphthalein) until a light pink colour is produced (p. 460). The endpoint is usually obtained quite sharply on adding the last one or two drops of standard alkali (two drops = 0·05 cc.). A correction is made for the original acidity of the alcohol.

Stage 3. *Titration in alcoholic formaldehyde.* After obtaining the reading in Stage 2, the same liquid is used for Stage 3. 12·5 cc. of aqueous formaldehyde solution prepared by diluting colourless formalin, with two volumes of distilled water, and neutralising the mixture to phenolphthalein, is added for each 50 cc. alcohol used in Stage 2, and the titration continued to the same endpoint as before. A mixture of neutralised diluted formalin and alcohol in the same proportions as they have been used is then titrated and the result obtained on completing Stage 3 is corrected accordingly. The acidity of rectified spirit is very slightly increased on adding the neutral formaldehyde solution.

The result obtained in Stage 1 gives useful information when dealing with the dibasic amino-acids, arginine, and salts of amino-acids (see p. 467).

The Stage 2 titration value includes the reading obtained in Stage 1. Several amino-acids are correctly estimated in Stage 2.

The increase in titration value obtained in Stage 3 gives an idea of the character of the amino-acid, and when dealing with an amino-acid mixture affords an indication of the amount of dibasic amino-acids and proline present. The carboxyl-groups of all the amino-acids contained in an amino-acid mixture, except that of arginine, are estimated by the total titration value obtained on completing Stage 3.

To avoid dilution of the alcohol, the use of $N/10$ alcoholic potash instead of $N/10$ soda was adopted. Since four drops of this reagent from an ordinary standard burette measure only 0·1 cc., a further advantage is gained in titrating. The alcoholic potash should be made from absolute alcohol and be free from carbonate. It should be as nearly colourless as possible and carefully guarded against evaporation.

The experiment with the basic methylene derivative of diethylamine showed that acid radicles can be titrated in the presence of such bases, if the concentration of alcohol is not less than 70 to 75 %. The addition of the stated amount of diluted aqueous formalin instead of alcoholic formalin was adopted at this stage, as it was found that sufficient formaldehyde was thus provided and at the same time the concentration of alcohol was not made lower than shown necessary in the experiment referred to. This slight modification proved more convenient.

The titrations should be done promptly in order to minimise any possible effect of CO_2 absorbed from the air.

Wider application of the method.

It will be evident from a study of the experimental results that the method outlined above is capable of much wider application in the following ways.

1. Aqueous solutions containing salts of ammonia, primary, secondary and tertiary amines and allied bases, either separately or as mixtures, can be investigated quantitatively, by means of Stage 2 of the method. The titration value thus obtained would represent the exact equivalent of the acid radicles present. If the original solution contained free acids as well as these salts, the result would include the equivalent of the free acids. The presence of free organic bases however would not influence the titration value.

2. In the case of mixtures of amino-acids and organic bases, the carboxyl-groups of the amino-acids, not including that of arginine, would be correctly estimated by the total titration value obtained on completing Stage 3 of the method. At the same time, the difference between the total titration value and the result of Stage 2, would give an indication of the content of dibasic amino-acids and proline, if present.

3. Animal products for example, muscle and organ extracts, faeces, urine, milk, blood, etc., vegetable extracts, and fluids containing the products of bacterial growth, may be investigated by the general method after treating in the manner described for preparing the "Alcoholic Extract" (p. 452). In many of these fluids, a mixture of organic acids, amino-acids, and organic bases occurs. The total titration value obtained on completing Stage 3 of the method would represent the total acid radicles present including the carboxyl-groups of the amino-acids, except that of arginine. The increase in titration value obtained by proceeding from Stage 2 and completing Stage 3 would approximately be proportional to the amount of dibasic amino-acids and proline present.

Experimental Section.

The experimental results illustrating the development of the new general method for estimating amino-acids will be given, as far as possible in the same order as they have been referred to in the foregoing section. Since Sörensen's formol titration method had been found to give very deficient results when titrating the acid radicles in salts of secondary amines, and to be unreliable in many cases when used for estimating amino-acids, each solution made up for the purpose of testing the new general method was also submitted to formol titration so that the results obtained by the two methods could be compared. Much difficulty was experienced in the formol titrations owing to the indefinite character of the endpoints (see p. 467). The light pink colour obtained sharply in titrating solutions by the new method was adopted as a standard and attempts were made throughout the formol titrations to obtain a colour of the same intensity. The results given in this section for all the titrations in alcohol or in alcoholic formaldehyde have been corrected for the original acidity of the alcohol or alcoholic formaldehyde.

The effect of alcohol upon the basicity to phenolphthalein of ammonia, primary, secondary and tertiary amines.

Standard aqueous solutions of the pure hydrochlorides of these bases, approximately *N*/10 in strength, were made up and 5 cc. or 10 cc. portions from each solution were titrated as described in Table III.

Table III.

Col. 1	2	3	4	5
Ref. No.	Substance	Formol titration *N*/10 alkali cc.	Titration in 85–90 % alcohol *N*/10 alkali cc.	Theoretical value of the acid radicle in the portion taken *N*/10 alkali cc.
1	Ammonium chloride	9·3	9·35	9·35
2	Ammonium sulphate	—	9·95	10·0
3	Monoethylamine hydrochloride	5·9	6·1	6·13
4	Dimethylamine hydrochloride	3·1 to 3·9	6·8	6·72
5	Diethylamine hydrochloride	0·85 to 1·0	9·02	9·13
6	Trimethylamine hydrochloride	0	4·72	4·6

Remarks. On adding the alcohol to the aqueous ammonium sulphate the salt separated. It was found that the separation could be prevented by previously dissolving 10 % glycerol in the alcohol. This mixture was neutralised before use. In the case of Nos. 4, 5 and 6 in Col. 4 the endpoints were especially sharp. The endpoints of Nos. 4 and 5 in Col. 3 were very indefinite. The figure for No. 6 in Col. 5 was determined by analysis.

Discussion. The results of the titrations in alcohol were practically quantitative in all cases.

Formol titrations of the salts of secondary amines showed that the basic methylene compounds were capable of neutralising the acid radicles to a large extent. The acid radicle of the tertiary amine salt possessed no formol titration value. It is interesting to note that methylene derivatives of secondary amines, *e.g.* $(C_2H_5)_2.N.CH_2.N.(C_2H_5)_2$ are tertiary amines, not sufficiently strong however to titrate quantitatively to phenolphthalein. This fact limits the usefulness of the formol titration method.

The effect of alcohol in different concentrations upon the basicity of trimethylamine.

5 cc. of the standard aqueous solution of the hydrochloride used in Exp. No. 6 of Table III was diluted with 5 cc. water. 97 % alcohol was added in small quantities at a time, and the liquid titrated with $N/10$ alcoholic potash after each addition. As too much alcohol had been added in the third increment a further 10 cc. water was added after taking the third reading. The readings were corrected for the original acidity of the alcohol, and the percentage of alcohol in the solution at the end of each titration was calculated.

The results given in Table IV are shown graphically in Fig. 1.

Table IV.

97 % alcohol cc.	none	1·0	3·5	13·5	23·5	33·5	43·5	53·5	63·5	78·5	103·5	153·5
Readings (corr.) $N/10$ alcoholic KOH cc.	0·02	0·07	0·5	2·78	4·19	4·5	4·59	4·67	4·73	4·78	4·78	4·78
% alcohol	0	9·4	28·0	43·8	56·7	63·7	68·7	72·4	75·2	78·3	82·1	86·2

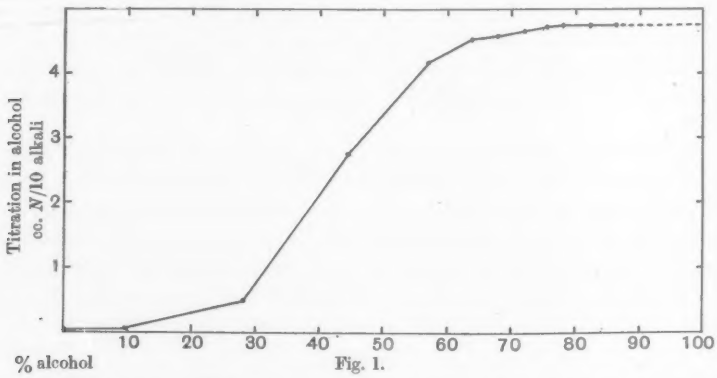


Fig. 1.

Discussion. The reading reached its highest point at 75–78 % alcohol, showing that at this concentration the free base is inactive to phenolphthalein. Similar results have been obtained on titrating ammonium chloride and the hydrochlorides of the other amines in the same way.

The effect of alcohol upon the basicity of the basic groups of amino-acids towards phenolphthalein.

The results of the experiments will be found in Table X, p. 466.

The effect of alcohol in different concentrations upon the basicity of the α-amino-group of amino-acids.

Experiment 1. 0·524 g. histidine monohydrochloride ($C_6H_9O_2N_3$, $HCl.H_2O$) was dissolved in water and made up to 50 cc. 10 cc. of the solution were neutralised to phenolphthalein with aq. $N/10$ soda. 5·1 cc. were required. Successive quantities of 97 % alcohol were then added, and the liquid titrated after each addition, exactly as described in investigating the basicity of trimethylamine. The results are given in Table V.

Table V.

97 % alcohol added cc.	1·0	2·0	3·0	4·0	5·0	10·0	15·0	20·0	30·0	40·0	50·0	75·0	100·0	150·0
Readings (corr.) $N/10$ alcoholic potash cc.	0·05	0·2	0·5	1·2	1·7	3·38	3·88	4·22	4·63	4·79	4·88	4·97	5·1	5·12
% alcohol	6·5	12·7	19·0	25·0	30·0	46·0	54·3	60·0	67·8	72·8	76·3	81·8	85·0	88·5

Remarks. The endpoints were not so distinct in the first five cases.

Discussion. In making up the solution the molecular weight of the salt was regarded as representing two equivalents of acid, one for the chloride radicle and the other for the carboxyl-group. The solution was made exactly $N/10$ on this basis. The chloride radicle in the pure salt titrated practically quantitatively in aqueous solution. According to Plimmer [1915] histidine is alkaline in reaction. The result does not support this statement so far as phenolphthalein is concerned.

The results show that the α-amino-group of histidine, liberated by the potash, behaved as ammonia and the amines to phenolphthalein in alcoholic solutions.

Experiment 2. 0·375 g. very pure arginine dinitrate ($C_6H_{14}O_2N_4$, $2HNO_3$) was dissolved in water and made up to 50 cc. The solution was thus exactly $N/20$ in relation to the $2HNO_3$. 10 cc. were neutralised with aq. $N/10$ soda to phenolphthalein. 2·5 cc. were required. 7·5 cc. water were then added and the experiment continued exactly as described in the case of the histidine hydrochloride. The results are given in Table VI.

Table VI.

97 % alcohol cc.	1	1½	2	3	4	5	10	15	20	30	40	50	100	150	200
Readings (corr.) $N/10$ alcoholic potash cc.	0·02	0·07	0·15	0·25	0·54	0·69	1·64	2·08	2·27	2·45	2·47	2·5	2·55	2·55	2·55
% alcohol	4·8	7·1	9·4	13·6	18·0	21·6	35·9	44·9	51·3	60·2	66·1	70·4	81·3	85·8	88·3

Discussion. As explained later (p. 468), on adding alcohol to the neutralised aqueous solution, the α-amino-group of arginine was liberated by the alkali from its internal combination with the carboxyl-group. The basicity of the free α-amino-group to phenolphthalein was completely submerged when the concentration of alcohol was increased to a sufficient extent.

The effect of different concentrations of alcohol upon the basicity to phenolphthalein of the basic methylene derivative of diethylamine.

The basic methylene derivative was made by adding 30 cc. neutralised diluted aqueous formalin (2 vol. water to 1 vol.) to 10 cc. of the standard aqueous solution of diethylamine hydrochloride used in the experiment No. 5 of Table III. The mixture was neutralised with $N/10$ soda to phenolphthalein, 1·0 cc. being required. Alcohol was then added in small quantities at a time and the experiment continued exactly as described in the case of the salts of trimethylamine, histidine and arginine. The results are given in Table VII.

Table VII.

97 % alcohol cc.	none	1·0	2	3	5	10	20	33	40	50	75	100	150	175
Readings (corr.) $N/10$ alcoholic potash cc.	1·05	1·2	1·5	2·39	4·09	6·38	7·96	8·53	8·77	8·85	9·05	9·15	9·2	9·22
% alcohol	2·56	5·1	7·9	11·7	18·2	28·5	40·3	49·8	53·7	58·1	66·0	71·2	77·7	79·9

Discussion. The acid radicle derived from the original amine salt titrated quantitatively when the concentration of alcohol was increased to 71 %. The basic methylene derivative, in common with ammonia, the amines and the free α-amino-group of amino-acids forms no ionisable compound with phenolphthalein when sufficient alcohol is present.

The low values given by certain amino-acids when titrated in alcohol are raised on adding formaldehyde to the neutralised alcoholic solutions.

The experimental evidence will be given in Table X, p. 466.

Effect of acetone upon the basicity of the basic groups of amino-acids towards phenolphthalein.

A thorough investigation of this question is proceeding. Up to the present only four of the amino-acids have been tested. Portions of standard aqueous solutions were first titrated with aq. $N/10$ soda to phenolphthalein. Acetone was then added, and the titrations continued. The results are given in Table VIII. Corrections have been made for the original acidity of the acetone.

Remarks. Glycine separates from aqueous acetone when the concentration of acetone reaches about 73 %. In Exp. No. 1, the glycine redissolved as the titration proceeded. Only a faint opalescence resulted on adding the acetone in Nos. 3 and 5. The results of Nos. 4 and 6 were obtained by continuing the titration after adding further acetone to the neutral liquids from Nos. 3

and 5 respectively. A small separation which remained in suspension was produced by the additional acetone in each case. This probably consisted of the sodium salts, as apparently the titration of the carboxyl-group was not disturbed.

In the case of proline about 0·2 cc. further soda was required on adding more acetone.

Table VIII. Preliminary experiments.

Ref. No.	Substance	Titration in aqueous solutions aq. $N/10$ soda cc.	Further aq. $N/10$ soda required on adding acetone cc.	Total titration value cc.	% acetone at the end of titration	Theoretical total titration value of portion taken cc.
1	Glycine	0·05	5·2	5·25	83·3	5·0
2	Proline	0·15	5·1	5·25	76·3	5·28
3	Aspartic acid	3·75	3·7	7·45	75·1	7·5
4	„ „	3·75	3·8	7·55	84·8	7·5
5	Glutamic acid hydrochloride	9·9	4·75	14·65	71·8	15·0
6	„ „	9·9	5·0	14·9	83·4	15·0

Discussion. In the case of glutamic acid a higher concentration of acetone than 71·8 % was required. The results show that the carboxyl-groups of these amino-acids titrate practically quantitatively if sufficient acetone is present. The general effect of acetone resembles that of alcohol.

The low titration values given by certain amino-acids in alcohol are raised on adding acetone to the neutralised alcoholic solutions.

Up to the present only aspartic and glutamic acids have been investigated. Portions of standard aqueous solutions were treated with the requisite amount of alcohol and titrated with aq. $N/10$ soda to phenolphthalein. Acetone was then added, and the titrations continued. The results are given in Table IX.

Table IX. Preliminary experiments.

Col. 1	2	3	4	5
Substance	Titration in alcohol, aq. $N/10$ soda cc.	Total titration value on adding acetone, $N/10$ soda cc.	% acetone at the end of titration	Theoretical total titration value of the portion taken cc.
Aspartic acid	6·3	7·6	28·1	7·5
Glutamic acid hydrochloride	12·65	14·85	45·0	15·0

In these two experiments the endpoint colour of the indicator showed a tendency to fade when obtaining the figures of col. 3. This phase extended over a range of about 0·2 cc. The fading was probably due to the effect of CO_2 absorbed from the air. This point needs further investigation.

The results show that to whatever cause the low titration values in alcohol are attributed (see p. 456) the addition of acetone or formaldehyde brings

about a disturbance of the equilibrium so that the total titration values are practically quantitative. It is evident that the action of these two reagents requires thorough investigation.

Estimation of carboxyl-groups of amino-acids by the new method.

Accurate $N/10$ solutions of the amino-acids were made up, the quantity necessary for the purpose in each case being calculated on the basis of the number of carboxyl-groups in the molecule. In the investigation of the amino-acid salts, the acid radicles as well as the carboxyl-groups were taken into account in the calculations. When dealing with mixtures, the calculation was based on the nitrogen content. A description of the specimens used and the modified procedure adopted for amino-acids of low solubility are given below:

1. Glycine pure.
2. Leucine 10·8 % N.
3. Phenylalanine, very pure, prepared from the pure hydrochloride.
4. Tyrosine, very pure. Three times crystallised.

0·0906 g. was dissolved in 7·5 cc. aq. $N/10$ HCl by dipping the vessel into hot water for a few seconds. After cooling, the requisite amount of alcohol was added, and the titration done promptly. Allowance was afterwards made for the acid used.

5. Cystine, very pure. Colourless hexagonal plates under microscope.

0·0601 g. was dissolved in 6 cc. cold aq. $N/10$ alkali. The right amount of alcohol was then added and the excess of alkali determined by titrating with $N/10$ HCl.

6. Tryptophan, very pure. Colourless glistening platelets.

0·102 g. was dissolved in 5 cc. water at about 85°, cooled, the alcohol added and titrated promptly.

7. Proline. Completely soluble in cold absolute alcohol.

Crystallised in desiccator. M.P. 216°. Contained only traces of impurity represented by a very small content of amino-nitrogen.

8. Asparagine. Large crystals.
9. Aspartic acid. Contained traces of insoluble impurity.

0·3328 g. dissolved in about 40 cc. hot water, cooled and made up to 50 cc.

10. Glutamic acid hydrochloride. Twice crystallised.
11. Histidine monohydrochloride. $C_6H_9O_2N_3$, $HCl.H_2O$. Very pure. M.P. 255–256°.
12. Arginine dinitrate $C_6H_{14}O_2N_4$, $2HNO_3$. Very pure.

Specimen completely freed from all traces of free HNO_3 by standing over potash in a desiccator for a long time, then thoroughly dried over $CaCl_2$. M.P. 145° (uncorr.) decomposing 150–160°.

13. Lysine picrate, pure recrystallised.

0·0938 g. dissolved in 10 cc. water by dipping vessel into hot water for a few seconds. Alcohol then added and the mixture titrated at once.

and 5 respectively. A small separation which remained in suspension was produced by the additional acetone in each case. This probably consisted of the sodium salts, as apparently the titration of the carboxyl-group was not disturbed.

In the case of proline about 0·2 cc. further soda was required on adding more acetone.

Table VIII. Preliminary experiments.

Ref. No.	Substance	Titration in aqueous solutions aq. $N/10$ soda cc.	Further aq. $N/10$ soda required on adding acetone cc.	Total titration value cc.	% acetone at the end of titration	Theoretical total titration value of portion taken cc.
1	Glycine	0·05	5·2	5·25	83·3	5·0
2	Proline	0·15	5·1	5·25	76·3	5·28
3	Aspartic acid	3·75	3·7	7·45	75·1	7·5
4	„ „	3·75	3·8	7·55	84·8	7·5
5	Glutamic acid hydrochloride	9·9	4·75	14·65	71·8	15·0
6	„ „	9·9	5·0	14·9	83·4	15·0

Discussion. In the case of glutamic acid a higher concentration of acetone than 71·8 % was required. The results show that the carboxyl-groups of these amino-acids titrate practically quantitatively if sufficient acetone is present. The general effect of acetone resembles that of alcohol.

The low titration values given by certain amino-acids in alcohol are raised on adding acetone to the neutralised alcoholic solutions.

Up to the present only aspartic and glutamic acids have been investigated. Portions of standard aqueous solutions were treated with the requisite amount of alcohol and titrated with aq. $N/10$ soda to phenolphthalein. Acetone was then added, and the titrations continued. The results are given in Table IX.

Table IX. Preliminary experiments.

Col. 1	2	3	4	5
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Aspartic acid	6·3	7·6	28·1	7·5
Glutamic acid hydrochloride	12·65	14·85	45·0	15·0

In these two experiments the endpoint colour of the indicator showed a tendency to fade when obtaining the figures of col. 3. This phase extended over a range of about 0·2 cc. The fading was probably due to the effect of CO_2 absorbed from the air. This point needs further investigation.

The results show that to whatever cause the low titration values in alcohol are attributed (see p. 456) the addition of acetone or formaldehyde brings

about a disturbance of the equilibrium so that the total titration values are practically quantitative. It is evident that the action of these two reagents requires thorough investigation.

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2. Leucine 10·8 % N.
3. Phenylalanine, very pure, prepared from the pure hydrochloride.
4. Tyrosine, very pure. Three times crystallised.

0·0906 g. was dissolved in 7·5 cc. aq. $N/10$ HCl by dipping the vessel into hot water for a few seconds. After cooling, the requisite amount of alcohol was added, and the titration done promptly. Allowance was afterwards made for the acid used.

5. Cystine, very pure. Colourless hexagonal plates under microscope.

0·0601 g. was dissolved in 6 cc. cold aq. $N/10$ alkali. The right amount of alcohol was then added and the excess of alkali determined by titrating with $N/10$ HCl.

6. Tryptophan, very pure. Colourless glistening platelets.

0·102 g. was dissolved in 5 cc. water at about 85°, cooled, the alcohol added and titrated promptly.

7. Proline. Completely soluble in cold absolute alcohol.

Crystallised in desiccator. M.P. 216°. Contained only traces of impurity represented by a very small content of amino-nitrogen.

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0·3328 g. dissolved in about 40 cc. hot water, cooled and made up to 50 cc.

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11. Histidine monohydrochloride. $C_6H_9O_2N_3$, $HCl.H_2O$. Very pure. M.P. 255–256°.
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Specimen completely freed from all traces of free HNO_3 by standing over potash in a desiccator for a long time, then thoroughly dried over $CaCl_2$. M.P. 145° (uncorr.) decomposing 150–160°.

13. Lysine picrate, pure recrystallised.

0·0938 g. dissolved in 10 cc. water by dipping vessel into hot water for a few seconds. Alcohol then added and the mixture titrated at once.

The results are given in Table X.

Table X.

Ref. No.	Substance	Col. 1	2	3	4	5	6
				New General Method			
			Titration in aqueous HCHO. Solutions not neutralised previously. aq *N*/10 soda cc.	Stage 1 Titration in water aq *N*/10 soda cc.	Stage 2 Titration in alcohol *N*/10 alc. potash cc.	Stage 3 Titration in alcoholic formaldehyde *N*/10 alc. potash cc.	Theoretical total titration value of the portion taken *N*/10 alkali cc.
	Amino-acids:						
1	Glycine		5·05	0·05	4·82	5·15	5·0
2	Leucine		4·3	—	4·7	5·1	5·05
3	Phenylalanine		4·65	—	5·0	5·02	5·0
4	Tyrosine		4·3	—	5·0	5·12	5·0
5	Cystine		4·87	—	5·0	5·05	5·0
6	Tryptophan		—	—	4·92	5·15	5·0
7	Proline		0·82	0·07	2·07	2·6	2·64
8	Asparagine		5·0	0·55	5·02	5·3	5·0
9	Aspartic acid		4·3	2·47	4·2	4·95	5·0
	Salts of Amino-acids:						
10	Glutamic acid hydrochloride		6·9	4·9	6·4	7·45	7·5
11	Histidine monohydrochloride		3·75	2·55	5·0	5·05	5·0
12	Arginine dinitrate		4·9	2·5	5·05	5·15	5·0
13	Lysine picrate		4·5	—	4·9	5·05	5·0
	Mixtures of Amino-acids:						
14	Glycine, alanine, leucines and valine		6·3	0·2	7·05	7·5	7·5
15	The monobasic amino-acids from the hydrolysis of caseinogen		2·32	—	2·75	3·0	?
16	Mixture of the same quantity of the solution used for No. 10 with half the quantity used for No. 15		—	—	8·12	9·05	9·0

Remarks. The titration values obtained in Stage 2 include those obtained in Stage 1.

In obtaining the results for Nos. 7, 9 and 10 in Stage 2, the faintest possible pink colour appeared at 1·75 cc., 3·8 cc. and 6·2 cc. respectively. No. 10 gave 6·6 cc. in Stage 2 when titrated hot.

The titrations of lysine picrate were difficult owing to the light brownish yellow colour of the solutions. Several readings were taken in the presence of an independent observer in the following manner: after each reading the liquid was made acid by adding a known quantity of *N*/10 HCl and again titrated with *N*/10 alkali. After allowing for the acid added in each case, the following results were obtained in succession:

Stage 2: 4·9, 4·95.

Stage 3: 5·05, 5·1, 5·05, 5·05. Colour too pronounced at 5·25 cc. No other pure lysine salt was available.

Picric acid was found to titrate quantitatively as a monobasic acid in

both Stages 1 and 2. Potassium picrate separated from the alcoholic solution on standing.

The mixture used in No. 15 consisted of the amino-acids recovered after removing alcohol-insoluble calcium salts from the hydrolytic products of caseinogen.

The results given in Table X may be summarised as follows:

Formol titration method.

Glycine and asparagine	quantitative results.
Cystine	only 2 % low.
Arginine (net result)	practically neutral.

In all other cases considerable deficiencies were shown.

New method.

Stage 1. Quantitative results were obtained for the following carboxyl-groups and acid radicles:

Dibasic amino-acids	One of the carboxyl-groups.
Histidine monohydrochloride ...	The chloride radicle.
Arginine dinitrate	One of the two nitrate radicles.

Stage 2. Phenylalanine, tyrosine, cystine, histidine, and asparagine gave quantitative titration values:

Tryptophan and lysine, practically quantitative.

Arginine (net result), quantitatively neutral.

Glycine, 3·5 % low.

Leucine and the mixture of glycine, alanine, leucines and valine, 6–7 % low.

Proline, 20–30 % low.

Aspartic and glutamic acids, 30–45 % low in relation to the value of one carboxyl-group.

Stage 3. Practically quantitative results in all cases.

Discussion. Formol titration method. In carrying out the titrations in aqueous formaldehyde, 30 cc. diluted formalin (1 vol. to 2 vols. water) neutralised immediately before use were used in each experiment. It was found necessary to dilute the formalin before it could be neutralised precisely to phenolphthalein. In a few cases the titration values were slightly increased on adding further neutral formaldehyde. When this happened the excess was made greater until the reading reached a maximum. No. 14 gave the highest increase obtained in this way, viz. by 0·35 to 6·65 cc. Tyrosine gave an increase by 0·2 cc. to 4·5 cc. In the other cases (including glycine) the increase was only about 0·1 cc.

Phenolphthalein was found to indicate very indefinitely in the formol titrations. Sörensen [1907] appears to have encountered the same difficulty, as he advocated the addition of 0·2 *N* alkali until a deep red colour developed, to be matched with a deep red colour prepared under known conditions in a control solution. The formol results given in col. 2 of Table X were obtained by titrating, until beyond all doubt the solutions were light pink in colour

(p. 460). If the opinion be held that the solutions are still acid, although a light pink colour has developed, then the indicator is unsuitable, and the results are unsatisfactory.

Abnormalities in the formol titration method are fully discussed by Jodidi [1918], who states that accurate results are given when the amino-acid molecule contains "amino- and carboxyl-groups *only*," and that if other basic groups, such as the imino-group, are present, the method is unsuitable. The results obtained in this present investigation confirm the observations of Jodidi save in regard to the monobasic monamino-acids, which all gave low titration values with the exception of glycine. Jodidi attributes the adverse influence of the imino-group to the effect of the basic methylene derivatives produced by the action of formaldehyde.

The new method. With the exception of aspartic acid, glutamic acid and proline, the endpoints of the titrations in alcohol (Stage 2) were quite satisfactory. Especially was this the case with those amino-acids which gave quantitative results. All the titrations in Stage 3 of the method gave satisfactory endpoints. The sharpness with which the light pink endpoint appears is well shown on titrating a little pure phenylalanine or trimethylamine hydrochloride in 90 % alcohol. If desired the endpoint so obtained may be used as a standard.

Arginine. In making up the standard solution of arginine dinitrate, the molecular weight of the salt was regarded as representing two equivalents in relation to the $2HNO_3$. The solution was made exactly $N/20$ on this basis. 10 cc. required 2·5 cc. $N/10$ alkali when titrated in aqueous solution, and double this quantity when titrated in alcoholic solution. The salt therefore behaved as a monobasic acid in water and as a dibasic acid in alcohol. This behaviour can only be explained by assuming that the guanidine nucleus is combined with one HNO_3 in the aqueous solution of the dinitrate, and that the other HNO_3 combines with an equivalent of alkali during the titration. In the neutralised aqueous solution therefore the α-amino-group and the carboxyl-group form an "internal salt." On adding the requisite amount of alcohol to the neutralised aqueous solution, the alkali equivalent of the carboxyl-group is required as in the case of other amino-acids. Consequently, the guanidine nucleus reacts as a univalent base to phenolphthalein in both aqueous and alcoholic solutions. This is the only nitrogenous base or basic group of those investigated up to the present, possessing the power of reacting with the indicator in alcoholic solution. Other guanidine derivatives and guanidine have been investigated under the same conditions. The results show that the reaction to phenolphthalein in alcoholic solution is a common property when the molecule contains a NH grouping attached to a carbon atom by a double bond. An account of this investigation will be given in another communication.

The neutrality of arginine to phenolphthalein in alcoholic solution distinguishes it from other amino-acids. The utilisation of this character as the

basis of a new method for estimating arginine in the hydrolytic products of proteins is under investigation.

Since the completion of the experimental work described in this paper, I found that Victor Birckner [June, 1919], when titrating grain extracts, noted that the acidity to phenolphthalein increased on adding alcohol to the aqueous solutions. The phenomenon was attributed to amino-compounds in the extracts. Birckner's main conclusions were summarised by him as follows: "Amino-acids which in aqueous solution are nearly neutral to phenolphthalein react distinctly acid in the presence of alcohol. This fact should be taken into account when making acidimetric titrations in alcoholic liquids containing amino-compounds such as various animal and vegetable extracts." The amino-acids Birckner tested were those which do not give quantitative results even in much higher concentrations of alcohol than he employed.

The fact that amino-acids become acid in alcoholic solutions was discovered quite independently and by an entirely different route.

Wider application of the method.

Further evidence of the quantitative character of the estimation of total acid radicles including the carboxyl-groups of amino-acids, by the new method, was obtained by titrating known mixtures of glycine and butyric acid. The results corresponded with the amounts known to be present.

The "Alcoholic Extract" which had been stored for several months, since the experiments of Table I were carried out, was again investigated by the method. 50 cc. required 23·37 cc. $N/10$ alkali in Stage 2 and 23·75 cc. in Stage 3. By comparing these figures with those given in Table I it will be noted that such preparations as the "Alcoholic Extract" can be preserved for a considerable time without incurring appreciable loss or change.

A definite quantity of the solution of the mixture of amino-acids used in Exp. No. 15 of Table X was added to a portion of the "Alcoholic Extract." The titration values obtained in Stages 2 and 3 were found to include the effect of the additional carboxyl-groups quantitatively.

It is evident that by the use of this method, data may be accumulated quickly in the investigation of many important problems, amongst which the following may be mentioned.

(1) The order of breakdown of the constituents of animal matter by the organisms of putrefaction, with a view to obtaining clues as to the best means of detecting the early stages of putrefaction in meat or fish, so that the quality of these materials may be gauged.

(2) The breakdown, separately in artificial media, of proteins, fats and carbohydrates by enzymes and bacteria. The action of pathogenic or other organisms in pure culture upon various sugars and amino-acids. Decarboxylation of amino-acids.

(3) Rapid estimations of organic acids in faeces with the object of throwing

light upon the work of organisms in the intestines as affected by unsuitability of rations or disproportion in the essential food constituents.

(4) Comparison of foods by experiments designed to imitate digestion.

(5) Investigation of extracts of agricultural products such as silage.

(6) Rate of hydrolysis of proteins, and the investigation of the order in which the amino-acids are split off.

(7) Investigation of the purity of specimens of amino-acids or their salts. It will be noted that the amino-acids are not destroyed during their investigation by means of Stage 2 of the method, and can therefore be recovered. When only small amounts of material are available this fact is of obvious importance.

Further investigations are proceeding in these directions.

The rapid estimations of volatile bases and amino-acids.

In the older method for estimating volatile bases and amino-acids in "Alcoholic Extracts" [Foreman and Graham-Smith, 1917], it was necessary to distil the bases at a low temperature in order to avoid the decomposing effect of the excess of alkali upon the amino-acids. A considerable amount of time and attention was therefore required in carrying out the distillations.

The total titration value of an aliquot portion of the "Alcoholic Extract," determined by completing Stage 3 of the new method, affords an exact measure of the amount of alkali necessary for liberating the bases from their salts. If no more than this amount be added a rapid method of distillation at a higher temperature can be adopted because decomposition of amino-acids is reduced to a minimum. The residual liquid becomes aqueous as the alcohol is removed and the alkali salts of the amino-acids hydrolyse, so that an amount of free alkali equivalent to the amino-acids is found in the aqueous solution.

An improvement in the titration of the volatile bases distillate is rendered possible by finding that a sharp endpoint can be obtained in all concentrations of alcohol when alizarin is used as an indicator. The endpoint is so sharp that one drop $N/10$ alkali in excess produces a very decided colour change. As the distillates can be titrated without adding water, a further advantage is gained by using alizarin instead of methyl orange.

The method used was as follows:

50 cc. "Alcoholic Extract" was placed in the flask of an ordinary steam distilling apparatus and the correct amount of $N/10$ alcoholic potash added. This amount had been ascertained in another portion by completing Stage 3 of the new method. A rapid current of steam derived from distilled water which had been freed from CO_2 by prolonged boiling, was passed through the solution until a foam nearly filling the flask developed. Air was admitted into the flask in which the steam was generated in time to prevent any of the foam from passing into the condenser. The foam usually appeared in four to five minutes. The steam inlet tube was then raised above the surface of the

liquid and the apparatus swept by a further current of steam for one minute. The bases with the alcohol were carried from the condenser to the receiver by a tube which dipped into a known quantity of $N/10$ acid. The excess of acid in the alcoholic contents of the receiver was determined by titrating with $N/10$ soda (standardised to alizarin) using alizarin as indicator. The amount of acid equivalent to the volatile bases was estimated by difference. The distillates need no dilution with water before titrating. The indicator can be added in the form of a weak aqueous emulsion, or in 0·5 % alcoholic solution.

The flask containing the residual liquid, which appeared to be quite free from alcohol, was cooled under the tap, the steam inlet tube washed with water and removed, and the solution titrated without delay with $N/10$ acid to phenolphthalein. Even if a very small amount of alcohol were present, its influence upon the titration value in the aqueous solution would be inappreciable (see Tables V and VI). The content of free alkali in the residual liquid represents approximately the difference between the total titration value of the carboxyl-groups of the amino-acids and the effect of those groups which titrate to phenolphthalein in aqueous solution.

By this method, the bases from three successive 50 cc. portions of the "Alcoholic Extract" neutralised 24·15 cc., 24·05 cc. and 24·15 cc. $N/10$ acid. The figure obtained by the older method several months earlier, for the same quantity of extract was 0·7 cc. higher. It was possible, however, for small losses to occur during the interval through exposing the extract to the air whilst removing aliquot portions. A further experiment showed that the whole of the volatile bases can be estimated in a single distillation. In one case 50 cc. alcohol were added to the residual liquid and the resulting solution steam distilled, as before, into a fresh quantity of $N/10$ acid. Only 0·1 cc. further acid was neutralised, making a total of 24·15 cc. for the two distillations.

The residual aqueous liquids consistently required 2·05 cc. $N/10$ acid for neutralisation to phenolphthalein. The figure obtained by the formol titration for the amino-acids several months earlier was 2·1 cc.

The effect of the process upon the composition of added amino-acids was tested. The complex amino-acid solution used in Exp. No. 15 of Table X was used for this purpose. A quantity possessing a total titration value of 3·0 cc. was added to 50 cc. "Alcoholic Extract," and steam distilled according to the method. The titration of the residual liquid gave a result 0·65 cc. too low, showing that a certain amount of change occurred during the short exposure of the added amino-acids to a temperature of 100°. It should be noted that the concentration of free alkali in the residual liquid is greater when more amino-acids are present. In this experiment the volatile bases result was the same as obtained previously, viz. 24·05 cc. It appears therefore that whatever change in the composition of the amino-acids occurred, volatile bases were not produced. Consequently the rapid method, which can be

completed in seven or eight minutes, seems to be very reliable so far as the volatile bases are concerned. Further attempts are being made to obtain an accurate separate figure for the amino-acids.

Summary and Conclusions.

1. Ammonia, primary, secondary and tertiary amines, and basic methylene derivatives of secondary amines, do not form ionisable compounds with phenolphthalein in alcoholic solutions containing water, if the concentration of alcohol is sufficiently high.

2. In aqueous alcoholic solutions of the salts of these bases the acid radicles can be titrated accurately with $N/10$ alkali, using phenolphthalein as indicator, if more than about 80 % alcohol is present.

3. When aqueous-alcoholic solutions of certain amino-acids containing about 85 % alcohol are titrated with standard alcoholic potash, the amino- or imino-groups liberated from their "internal salt" combinations, resemble ammonia and the amines in showing no basicity to phenolphthalein, and the carboxyl-groups are accurately estimated.

4. Other amino-acids, more particularly dibasic amino-acids and proline, give low results when titrated in alcohol under these conditions, possibly owing to loose combination of alcohol with a carboxyl-group, or loose condensation. The subsequent addition of formaldehyde or acetone, however, results in a disturbance of the equilibrium, so that the carboxyl-groups titrate quantitatively.

5. The effect of acetone upon the basicity of the basic groups of amino-acids to phenolphthalein is similar to that of alcohol. The amino-acids tested up to the present have all given practically quantitative results when titrated with $N/10$ alkali in aqueous acetone containing 80–85 % acetone.

6. The guanidine nucleus of arginine behaves differently from other nitrogenous bases or basic groups by titrating quantitatively as a univalent base in alcohol as well as in water. An explanation of this unique behaviour will be given in a further communication. Arginine is neutral to phenolphthalein in alcoholic solutions if sufficient alcohol is present, the carboxyl-group and the guanidine nucleus exactly neutralising one another.

7. A simple general method for accurately estimating the carboxyl-groups of amino-acids, based upon the foregoing conclusions, is described.

8. It has been shown further that the method is capable of much wider application. The total acids (including the carboxyl-groups of amino-acids) whether in the free state or combined with organic bases, can be accurately estimated in alcoholic preparations made from aqueous fluids such as those which contain the products of bacterial growth.

As the titration value is unaffected by the free bases their removal is

unnecessary. Consequently the solutions need no heating, and there is no risk of decomposition.

9. A rapid method for estimating volatile bases is also described.

Finally I wish to thank Mr C. E. Bryant for assistance in carrying out the experiments and for acting as an independent observer of the endpoints of the titrations.

REFERENCES.

Birckner (1919). *J. Biol. Chem.*, **38** [No. 2, June], 245.
Foreman and Graham-Smith (1917). *J. Hygiene*, **16**, 144.
Jodidi (1918). *J. Amer. Chem. Soc.*, **40**, 1031.
Mann (1906). *Chemistry of the Proteids*, p. 211.
Plimmer (1915). *Practical Organic and Bio-Chemistry*, p. 285.
Sörensen (1907). *Biochem. Zeitsch.*, **7**, 79.
Sutton (1900). *Volumetric Analysis*, 8th edition, p. 38.
Walker (1904). *Zeitsch. physikal. Chem.*, **49**, 82.

XXXIX. ADSORPTIVE STRATIFICATION IN GELS. IV.

By SAMUEL CLEMENT BRADFORD.

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(With Plates VII and VIII.)

Direct proof has already been given [Bradford, 1920] of the fundamental rôle of adsorption in the formation of banded precipitates. Unfortunately, owing to its extremely complex nature, mathematical analysis of the phenomenon appears impossible. The adsorbing surface at each band is affected by the mass of the precipitate and by the various factors of von Weimarn's formula, which vary, in an intricate way, with the concentrations of the reaction components. Moreover, notwithstanding statements to the contrary, the diffusion of the hypertonic reagent appears, at times, to be considerably affected by the mass and structure of the precipitate through which it has to percolate. In this connection it is sufficient to compare the extreme slowness of the diffusion of $N/5$ Pb $(NO_3)_2$ solution into $N/20$ K_2CrO_4 agar gel, with the rate of diffusion into $N/200$ K_2CrO_4 agar gel of the same solution, which reaches the base of the tube in 14 days. As in the case quoted, band formation does not seem to occur unless the precipitate allows of ready diffusion of the hypertonic reagent. This may be one reason why very colloidal precipitates do not form good bands. But this effect may also be due to the sudden separation of too large a mass of precipitate at one time, owing to excessive supersaturation. The complexity of the phenomenon, generally, is further increased by the influence of the reaction components on the formation of the precipitate, which was first pointed out by Bechhold [1905], and is confirmed in a remarkable way by the experiments to be described. It appears hopeless, therefore, at present, to make exact deductions which can be tested quantitatively.

The precipitate, produced by the interaction of an aqueous reagent on a gel containing a suitable solute, will create a solid boundary surface between the reagent and the solute, at which, by Gibb's law, the concentrations of both dissolved substances will usually increase. If the aqueous reagent is hypertonic to the gel, its concentration at the solid surface will be in excess of that of the solute in the gel, and the reagent will be able to diffuse into the gel. With a hypotonic aqueous reagent the adsorption of the gel solute will usually be greater, so that the aqueous reagent will be precipitated, as it collects at the solid surface, and will be unable to pass into the gel. This is Pringsheim's law. The result, however, depends on the formation of a precipitate. Hitherto, it does not always appear to have been realised that, in

the absence of a precipitate, diffusion can take place in both directions. Coloured reagents make this apparent. When strong ammonium hydroxide is poured on a gel containing copper sulphate the deep blue colour gradually diffuses both ways, until, after 48 hours, the solution and the gel have become the same colour throughout.

Since the separation of the strata depends on adsorption of the gel solute from the region between, it follows that the concentration of the hypotonic reagent must be of primary importance. The distance between the bands should usually be affected more by variations in the concentration of the gel solute than of that of the aqueous reagent. Moreover, adsorption experiments with coloured reagents indicate that the concentration of the gel solute in the direction away from the precipitate may increase very slowly at first, and then quite suddenly, as the maximum concentration of the gel is approached. This would tend to eliminate the influence of the valency of the reaction components in the metastable product, since the critical value, at which precipitation takes place, would be reached at approximately the same point where the sudden rise in concentration occurs. For the same reason this narrow region is the only place where any kind of supersaturation should happen.

These conclusions are subject to modification on account of the inhibiting, or precipitating, action of the various ions present, but they are fairly well borne out by experiment. Four tubes containing: (1) $N/80$ $CaCl_2$ agar gel, (2) $N/50$ $CaCl_2$ agar, (3) $N/70$ Na_2CO_3 agar and (4) $N/20$ Na_2CO_3 agar, treated with: (1) and (2) $2N/1$ Na_2CO_3 and (3) and (4) $N/10$ $CaCl_2$ solutions gave bands at distances (cm.) apart as follows:

(1)	(2)	(3)	(4)
0·57	0·35	0·65	0·15
0·73	0·45	0·65	0·22
1·00	0·55	0·85	0·225
		0·95	0·225
			0·275
			0·225
			0·50
			0·40
			0·55

For a given pair of reagents the figures are roughly inversely proportional to the concentration of the gel.

In another set the tubes contained: (1) and (2) $N/30$ KI agar and (3) and (4) $N/60$ KI agar. These were treated with: (1) and (3) $N/1$ $Pb(NO_3)_2$ solution and (2) and (4) $N/2$ $Pb(NO_3)_2$ solution. In this case halving the strength of the hypertonic reagent increased the distances of the bands only very slightly, while a corresponding change in the hypotonic reagent caused an approximate doubling of the distance apart of the strata. The inversely proportional effect comes out although the ionic product contains the square of the concentration of the hypotonic reagent.

In a third series the tubes contained: (1) and (3) $N/100$ K_2CO_3 agar and (2) and (4) $N/200$ K_2CO_3 agar. On these were poured 10 cc. each of: (1) and (2) $N/5$ Pb $(NO_3)_2$ and (3) and (4) $N/10$ Pb $(NO_3)_2$ solutions. The following curves, Fig. 1, were drawn freely through points obtained by plotting the distances apart of the bands, as ordinates, against the distances below the surface of the gel of the mid-points between the bands, as abscissae.

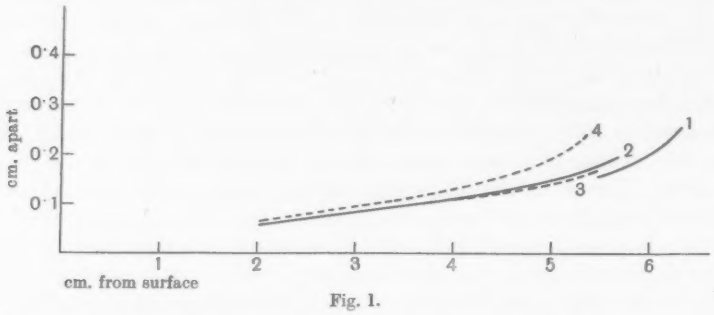


Fig. 1.

The very slight difference caused by halving the strength of the hypertonic reagent is apparent, as compared with the much greater effect of a similar change in the gel solute.

Similar reasoning shows that different effects should be obtained according to which reagent of a pair is dissolved in the gel, or to the actual solute chosen to provide a given reaction component. For example bands of lead carbonate should depend on whether the lead salt or the carbonate is dissolved in the gel, and on the particular soluble lead salt and carbonate used. 1 % agar gels containing: (1) $N/100$ K_2CO_3, (2) $N/100$ $Pb(NO_3)_2$ and (3) $N/100$ Na_2CO_3 were treated with: (1) and (3) $N/10$ $Pb(NO_3)_2$ and (2) $N/10$ K_2CO_3 solutions. The remarkable differences in the bands are shown in Figs. 1, 2 and 3, Pl. VII. The very fine lamination in (2) does not show clearly in the figure. With sodium carbonate as hypotonic reagent the bands are noticeably less regular than with the potassium salt.

It has already been shown [Bradford, 1918] that, since adsorption must depend directly on the specific surface of the precipitate, the formation of bands is determined by the value of N in von Weimarn's formula

$$N = K.\frac{P}{L}.$$

Recently, this expression has been criticised by Buchner and Kalff [1920]. Their experiments show that, in a given case, the dispersity of the precipitate is proportional to P/L, that is

$$N \propto \frac{P}{L}.$$

They contend, however, that the factor K in von Weimarn's formula is not directly proportional to the viscosity of the reaction medium and to the

degrees of aggregation, chemical (= complexity of structure) and physical, of the reaction components. Thus they obtained precipitates in the gel state with values of P/L varying from 75, 140 and 360 in the case of BaF_2, $CaSO_4$ and PbI_2, to about 10^4, 10^6, 10^7 and 10^8 for $BaSO_4$, AgCl, AgBr and AgI respectively, and they argue that, while chemical complexity has no exact meaning, the physical aggregation and viscosity cannot be so great as to account for these differing values.

That the chemical complexity of the molecule cannot be ignored is shown by glancing through the pages of Beilstein where its undoubted influence on the form in which a substance occurs may easily be traced. There is, however, an important factor which influences the value of P, which does not seem to have been taken into account hitherto. This is the amount of mixing of the reagents which happens before a precipitate is formed. It can easily be shown that in the case of a substance such as PbI_2, for example, where

$$H = Pb^{\cdot\cdot} \times I'^2,$$

the maximum value of P occurs when two volumes of the iodide solution mix with one volume of the solution of lead salt. In a given case, the degree of mixing before precipitation would certainly be affected by the metastable limit as modified by the various reaction components. The extraordinary effect of H on the form of the precipitate is shown by the experiments with the silver chromates which follow. Thus it will be seen that the value of P may easily differ greatly from that calculated on the assumption of immediate mixing of equivalent volumes of the reacting solutions. It seems probable that the remarkably different values of P/L found by Buchner and Kalff may be due, very largely, to variations in the corresponding metastable limits.

It must further be noted that L is not the normal solubility of the given substance, but its solubility under the actual conditions of the experiment. Again, the permanency of form of the precipitate may be influenced by the presence of the reaction components, which may tend to produce double salts, or otherwise to peptise the precipitate.

However, these considerations emphasise the difficulty of evaluating the factors in von Weimarn's formula, and it might be preferable to write it

$$N = U.\frac{P}{L},$$

where U is a function, as yet undetermined, of the viscosity of the medium and of the size and structure of the particles in solution, whether due to physical aggregation or chemical complexity. That is to say the formula is, as yet, an incomplete expression of the truth. It is, nevertheless, most useful as a working rule.

It will be seen, therefore, that banded precipitates can be produced only when the specific surface of the precipitate is great enough to cause the exhaustion of the solute from the neighbouring region of gel. Decrease in

N leads to diminution in adsorbing surface until stratification ceases. On the other hand, increase in N may be accompanied by increase in density of the precipitate and slower diffusion of the precipitant through the column of precipitate, until the precipitant cannot permeate the precipitate fast enough to traverse the exhausted region of gel immediately beyond before this is replenished with solute by diffusion from the gel. This is often noticed with lead salts, which, frequently, do not form strata unless the hypotonic reagent is very dilute, usually about $N/200$. When the gel solute is too concentrated, a dense, continuous band of precipitate grows until all the solute has been exhausted from the gel. This generally happens long before the column has reached the base of the tube, as in Fig. 1 of a previous paper [Bradford, 1920] which shows a column of lead chromate nearly complete.

Since the solute in the gel must be sufficiently dilute to allow of its exhaustion from the neighbourhood of the precipitate, with corresponding limitation of the value of P, it is clear that stratification can take place only when L is very small, or when K is large enough to compensate for a somewhat larger value of L. This is why only the more insoluble precipitates readily yield banded structures, and these only within comparatively narrow limits of concentration.

Although it has been argued that the adsorption theory leaves the gel out of account altogether [Hatschek, 1919], it will be seen that the above considerations are sufficient to explain the extraordinary differences in the form of a given precipitate when produced in different gels. The reaction medium, by its viscosity and protective action, profoundly modifies the values of K and P, and, thereby, the specific surface of the precipitate. The nature of the gel is, therefore, of fundamental importance. Various examples of the effect of the gel on the structure of the precipitate have already been discussed [Bradford, 1918]. Two of the most striking cases, and, perhaps, the most well-known, are those of the chromate and dichromate of silver, which form beautiful brownish red bands in ordinary gelatin gels and yield in agar only macroscopic black crystals. It was pointed out, that the absence of stratification in agar must be due to the small specific surface of the precipitate, and that, by increasing this, it should be possible to produce bands in agar similar to those in gelatin. This has been completely realised by experiment. It further transpires that the original Liesegang phenomenon in ordinary gelatin is due to the presence of impurities.

Fig. 6, Pl. VIII, shows the result of treating $N/20$ $K_2Cr_2O_7$ 1 % agar gel with $N/2$ $AgNO_3$ solution. It is a good illustration of the effect of slow diffusion in producing large crystals of a substance which occurs as a finely divided precipitate when the reacting solutions are quickly mixed. The formation of macroscopic crystals of silver dichromate shows that the number of crystallisation centres, N, is much too small. The only criterion for the production of bands is a sufficient increase in N. The application of this principle provides a crucial test for the adsorption theory.

A difficulty in the experimental work is the narrowness of, frequently, both the concentration limits, between which good bands are obtained. For this reason it is possible to make a series of experiments with substances, capable of forming beautiful strata, without obtaining any.

The first experiments were directed towards increase of specific surface by variation in the concentration of the reaction components, or the solubility of the precipitate. The following table shows the result of diminishing the strength of both reagents.

Reagent in the 1 % agar gel	Aqueous reagent	Result
$N/50$ $K_2Cr_2O_7$	$N/10$ $AgNO_3$	Red surface layer, clear space 0·25 mm., then small crystals with suggestion of stratification.
$N/75$ $K_2Cr_2O_7$	$N/10$ $AgNO_3$	Red surface layer, small crystals arranged distinctly in bands at 1, 4 and 10 mm. below surface.
$N/100$ $K_2Cr_2O_7$	$N/20$ $AgNO_3$	Red surface layer, band of microscopic black crystals at 5 mm., part of a red band at 14 mm.
$N/200$ $K_2Cr_2O_7$	$N/20$ $AgNO_3$	Red surface layer, band of very small crystals at 1 mm., wide dark red turbidity from 12·5 to 27 mm.
$N/300$ $K_2Cr_2O_7$	$N/20$ $AgNO_3$	Red surface layer, band of very tiny crystals at 1 mm., and larger particles from 3 mm.

As the concentrations are reduced, the particles of precipitate grow smaller, with corresponding increase of specific surface, and the tendency to produce bands begins to make itself manifest. The crystals were, however, still much too large. The addition of alcohol further diminished the size of the particles and increased the formation of bands. $N/20$ $K_2Cr_2O_7$ agar gel containing 30 % of absolute alcohol, treated with $N/10$ $AgNO_3$ solution, gave a precipitate in tiny feather-like crystals in distinct layers at 0·5, 2·0 and 3·5 mm. below the red surface layer.

Since the aqueous phase in gelatin gels contains about 0·3 % of gelatin, the effect of adding small quantities of gelatin and gum arabic was tried in a great many experiments. Many of these gave short columns of red precipitate instead of merely surface layers, but after about 1 cm. the colour of the column changed to black with the development of distinct crystals or spherites. With the aid of a lens, however, the red columns were frequently seen to be laminated. By increasing the concentration of the silver nitrate solution to $N/2$, two separate red bands were obtained in $N/75$ $K_2Cr_2O_7$ $\frac{1}{4}$ % agar gel containing 0·5 % of gelatin. Numbers of very beautiful black spherites of $Ag_2Cr_2O_7$ with a greenish lustre were formed below the bands, reaching 0·5 mm. in diameter at the base of the tube. With $2N/1$ $AgNO_3$ solution and $N/175$ $K_2Cr_2O_7$ 1% agar gel containing 0·06 % gelatin, five bands were formed, the first two red, those below composed of microscopic black spherites. These experiments appeared to show that the red precipitate is not due entirely to the effect of the gelatin. While the column of precipitate remained red it showed very similar banding to the commencement of a column in gelatin. The difficulty appeared to be that the specific surface diminished, as the reagents became more dilute by diffusion, until stratification ceased.

It seemed desirable to find some other way of increasing N. Remembering Bechhold's experiments on the effect of ions on the precipitate, Liesegang's [1914] practice of adding citric acid to washed gelatin was considered. It seemed probable that the trivalent anion of citric acid might tend to prevent the precipitation of negatively charged silver chromate particles, thus increasing the supersaturation and giving a much greater value of P. A series of experiments from this point of view showed that there is a definite concentration of citric acid, or of soluble citrate, for each concentration of chromate, or of dichromate, in the gel, which gives perfectly formed strata with clear interspaces.

Figs. 7 to 12, Pl. VIII, show the effect of adding increasing amounts of citric acid. As the particles grow smaller, the banding gradually appears. At first, while the crystals are still large enough to appear black, only a few layers are formed close together as seen in Figs. 7 and 8. With more citric acid a number of skeletal bands of red precipitate appear, which are much wider apart, but which break up into black spherites lower down the tube, Fig. 9. With the optimum amount of citric acid the red bands are complete and extend right down the tube, Fig. 10. Further increase of citric acid yields a more colloidal precipitate, at first irregularly stratified, Fig. 11, but ultimately separating in large flocks with little trace of stratification, as in Fig. 12. In Figs. 7 to 10 the effect was obtained by decreasing the concentration of potassium chromate while keeping the citric acid constant at $N/300$. The tubes contained respectively $N/88$, $N/107$, $N/125$ and $N/150$ K_2CrO_4. Fig. 11 had $N/140$ K_2CrO_4 with $N/250$ citric acid, and Fig. 12 $N/100$ chromate with $N/130$ citric acid. The optimum concentration of citric acid for $N/150$ K_2CrO_4 is $N/300$. A similar series was obtained with $N/100$ K_2CrO_4 agar gel by varying the amount of trivalent anion. The optimum concentration was found to be $N/200$. With $N/75$ K_2CrO_4, $N/125$ citric acid gave the best results.

The agar gel was made by soaking shredded agar for 24 hours in sufficient water to make a 1·25 % gel, afterwards boiling till dissolved and filtering through a Buchner funnel with washed linen. The gel was stored in wide-mouth bottles containing a trace of camphor. The test-tubes used were roughly graduated at 3 cc. and 15 cc. The required amounts of standard solutions of the reagents were added to make the gel of the desired strength, and the liquid made up to the 3 cc. mark with water. The hot agar sol was then filtered into the tube up to the 15 cc. mark, the whole mixed and allowed to set by placing in cold water. This gave a 1 % agar gel. To each tube 3 cc. of $2N/1$ silver nitrate solution were added, very carefully, from a pipette with its nose just above the top of the gel. Without great care, or if the tube was afterwards slightly shaken, the silver solution was liable to run down between the gel and the tube. The standard solutions of K_2CrO_4 and citric acid were made from recrystallised materials, the former by weighing and the latter by titration with carbonate-free alkali and phenolphthalein. It was remarkable that with ordinary stock reagents very different relative amounts of chromate

and citric acid were required. This was assumed to be due to traces of impurities.

The bands produced with citric acid and potassium chromate in agar are much wider apart than in gelatin, and, as they are almost free from secondary white precipitate, the effect is very beautiful. Moreover, they begin to form immediately the silver solution is poured on the gel, which makes them particularly suitable for demonstration. The same recipes give good results in plate preparations and in filter paper.

The effect of citric acid in gelatin was attributed by Liesegang to the hydrogen ion. This does not appear to be the case, since equally beautiful results are obtained by the use of potassium citrate. The bands are now very close together, almost exactly like those in gelatin, but perhaps closer. After about 10 mm. they become granular and blurred, suddenly changing into more colloidal, thicker and wider bands at the base of the tube, just as silver chromate does in gelatin. The optimum concentration for $N/150$ K_2CrO_4 was $N/85$ potassium citrate. By substituting citric acid for part of the potassium salt, the bands became wider apart and lost the blurred granular and more colloidal regions as in Fig. 5, Pl. VII. Wonderfully beautiful bands are produced with potassium citrate and potassium dichromate, which resemble, very closely, those formed in washed gelatin with citric acid. Fig. 4, Pl. VII, shows a 1 % agar gel with $N/100$ $K_2Cr_2O_7$ and $N/55$ potassium citrate and $2N/1$ $AgNO_3$ poured on top. It must be noted, however, that these reagents were not specially pure, or less citrate might have been necessary.

Bearing in mind that the chromates of silver do not form bands in washed gelatin, it seems evident that the different effects obtained with these salts in commercial gelatin and in agar gels are due, rather to mineral impurities contained in the gelatin, than to differences in the action of the gel-substances themselves. This raises the question as to whether the great protective action of commercial gelatin is a property of the pure substance.

The action of the citrate ion in these experiments is artificially to increase the supersaturation of the silver chromate before precipitation takes place, giving a larger P and greater specific surface of the precipitate. By actual cataphoresis, in the presence of gelatin and the reaction components, Mr Hatschek very kindly confirmed the hypothesis that silver chromate particles are negatively charged. This supports the view that the increased supersaturation is due to the trivalent anion. The inhibiting effect of citric acid was demonstrated by experiments on the mutual precipitation of dilute aqueous solutions of K_2CrO_4 and $AgNO_3$ of varying strengths in the presence of different amounts of citric acid. One reagent was added slowly, drop by drop, with vigorous stirring, to the other, mixed with citric acid, until sudden precipitation of the whole excess of silver chromate took place. A number of curves have been obtained showing the relation between the metastable limit and the concentration of citric acid, for different strengths of the reacting solutions. The experiments show that citric acid enormously increases the critical value of the ionic product for silver chromate at which spontaneous precipitation

takes place. Taken in conjunction with Bechhold's observations on the influence of ammonium nitrate in the same reaction, it seems extremely probable that aqueous supersaturated solutions are affected by electrolytes in the same way as colloid solutions. The experiments are being continued.

These investigations must not be taken as supporting the "supersaturation" theory of band formation. It has been shown that the reason why the silver chromates do not form bands in agar or washed gelatin is merely that the particles of precipitate are too large. Precipitates with small specific surface never form bands. The experiments show that, as the size of the particles diminishes, the tendency to stratification appears. The use of soluble citrates is merely a convenient way of increasing the dispersity of the precipitate. To leave no doubt on this point it seemed desirable to continue the experiments in which the specific surface was increased by diminishing the solubility of the precipitate. This time potassium chromate was used, as silver chromate is less soluble than the dichromate. It was immediately found that $N/100$ K_2CrO_4 0·7 % agar gel, containing 30 % of absolute alcohol, treated with $2N/1$ $AgNO_3$ solution gave a good series of narrow bands of Indian red silver chromate, which were very similar to those in ordinary gelatin, but so close together as to require a lens to see them clearly. In this case the precipitate was distinctly granular, like those of lead chromate and iodide. It is clear, therefore, that increased supersaturation is not necessary for the formation of bands. The success of these experiments affords seemingly conclusive evidence for the adsorption theory.

Summary.

Further evidence has been adduced to show that the formation of banded precipitates is due to adsorption of solute from the region between the bands.

Only precipitates with great specific surface form bands.

The specific surface of the precipitate is influenced by the reaction medium, by the presence of ions and particularly of trivalent ions.

By varying its specific surface, a given substance can be obtained in the banded form, or not, as desired.

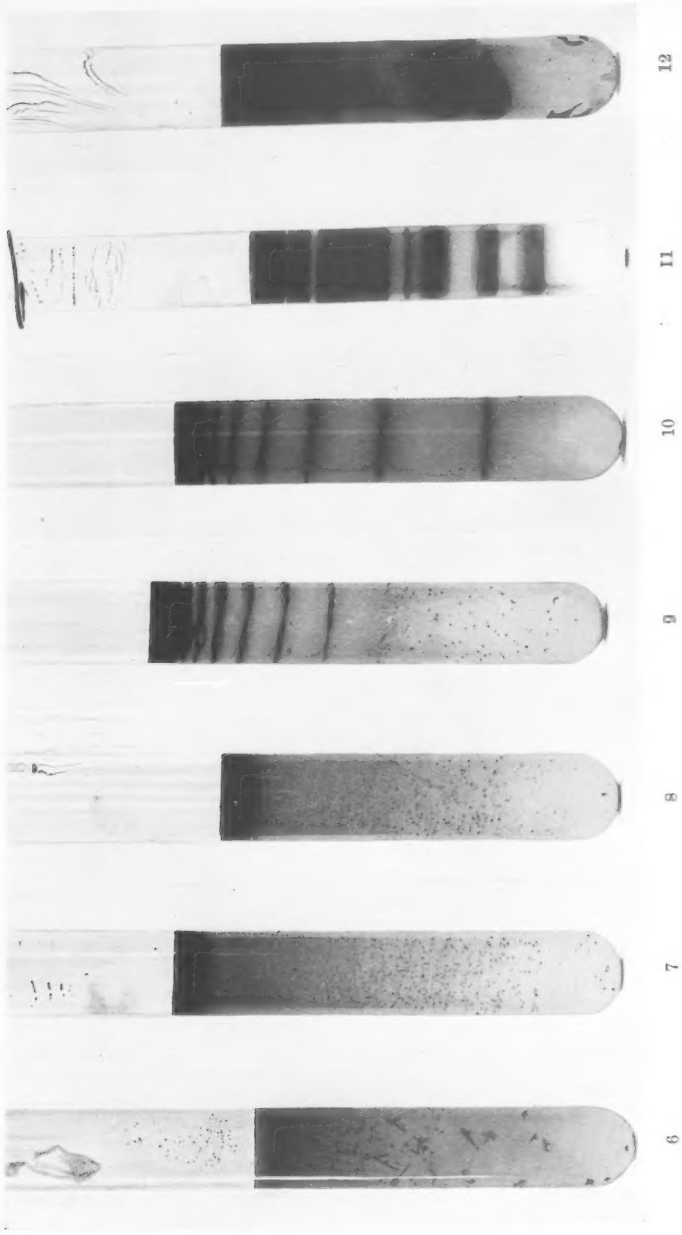
The occurrence, or non-occurrence, of bands of the same substance in different gels is due to the influence of the reaction medium on the dispersity of the precipitate.

These principles have been tested by application to the well-known cases of silver chromate and dichromate which form bands in gelatin gels and not in agar. The non-occurrence of bands in the latter medium is due to insufficient specific surface of the precipitate. By increasing the dispersity, beautiful bands of both substances have been obtained in agar gels.

REFERENCES.

Bechhold (1905). *Zeitsch. physikal. Chem.*, **52**, 185.
Bradford (1918). *Biochem. J.*, **11**, 14.
—— (1920). *Biochem. J.*, **14**, 29.
Buchner and Kalff (1920). *Rec. Trav. Chim.*, **39**, 135.
Hatschek (1919). Rep. Brit. Assoc., 1918, *Colloid Chemistry*, 21.
Liesegang (1914). *Zeitsch. physikal. Chem.*, **88**, 1.

N/10 Pb(NO3)
N/100 K2CO3
15
N/10 K2CO3
N/10 Pb(NO3)
14
N/10 Pb(NO3)
N/100 Na2CO3
16
1
2
3
4
5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XL. THE NATURE OF YEAST FAT.

By IDA SMEDLEY MacLEAN and ETHEL MARY THOMAS.

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Comparatively little is known of the nature of the fat present in simple cellular organisms and of the processes by which it is formed. It is possible that a study of such organisms, where the conditions of growth are easier to control, may throw more light on the method by which fat is formed than investigations of the more complex plants and animals. Since yeast is obtainable in quantity, it was chosen as the most suitable material for such an investigation; as a preliminary it was necessary to identify with certainty the constituents of yeast fat.

Characteristics of Yeast Fat.

The fat examined was obtained from the following sources:

(1) Baker's yeast supplied from the manufacturers.

(2) A pure culture of a brewery yeast grown for us under definite conditions by Dr Thaysen at the bacteriological laboratory of the Royal Naval Cordite Factory.

(3) Specimens of brewery yeast.

Method of Extraction of the Fat.

The pressed yeast was spread in a thin layer and dried in the hot room at 37° for 48 hours[1]. It was then soaked over night in alcohol and shaken for eight hours at ordinary temperature. The second and third extractions were similarly carried out, a mixture of alcohol and light petroleum (B.P. 60–80°) being used as the solvent. After the third extraction, 25 g. of the dry residue were extracted for eight hours in a Soxhlet apparatus with ether. The amount of fat obtained in the final extraction was always very small and was used only for calculating the total amount of fat. The alcohol and petroleum were evaporated on a water bath under diminished pressure and the residue dissolved in light petroleum and filtered. After evaporation of the solvent, the residue was dissolved in alcohol and excess of acetone added to precipitate the lipins. The solvent was distilled from the filtered solution; the iodine and saponification values were determined and the fat converted to the methyl esters, or saponified for the preparation of the sterols.

[1] Recently, the preliminary drying at 37° has been discarded since a considerable proportion of the fat seems to disappear during the process.

The crude yeast fat prepared by extraction of the alcohol-soluble residue with ether or with light petroleum consists of a brown liquid from which crystals of a sterol separate on standing. It has a high iodine value and a low saponification value attributable to the large proportion of unsaponifiable matter present which may be from 25 to 45 %.

Iodine Value.

The iodine value was determined by Wijs' method after precipitating the lipins by acetone. Twelve specimens grown from a pure culture of brewery yeast by Dr Thaysen gave values lying between 147 and 175·5, the average being 161·2. Four specimens of brewery yeast obtained locally gave an average value of 164·4. Six specimens of baker's yeast gave values from 121·2 to 150·8, the average being 135·6.

The iodine value therefore varies within wide limits. We found that this was dependent chiefly on the amount of sterol present in the crude fat. The yeast sterol has a very high iodine value and gives quite erratic results when the latter is determined by Wijs' method (see p. 490).

Saponification Value.

This is always low and shows wide variation, depending on the proportion of sterol present. Nine specimens of brewery yeast obtained from Dr Thaysen gave an average value of 162·3, the numbers lying between 138 and 184; four specimens of brewery yeast obtained from a brewery gave an average value of 164. The values from baker's yeast were higher, three specimens from one firm giving values from 196 to 199, while other specimens gave values of 187 and 151.

We endeavoured to determine the proportion of sterol in a specimen of crude fat obtained from baker's yeast, and in the unsaponifiable matter derived from it, by precipitation with digitonin solution [Windaus, 1909]. The result shows that the greater proportion of the sterol seems to be present not in the free state but as fatty acid ester.

In one experiment, the proportion of unsaponifiable matter was determined by extracting the solution with ether after it had been saponified and drying the residue in vacuo at 100°.

0·5456 g. fat gave 0·2175 g. unsaponifiable matter.

The unsaponifiable residue from a similar quantity of fat was dissolved in 50 cc. 95 % alcohol and a solution of 0·5 g. digitonin in 50 cc. of 90 % alcohol added. The resulting precipitate was filtered through a Gooch crucible, washed with 95 % alcohol and dried at 110°.

Weight of digitonin cholesteride	0·3763 g.
Solubility allowance	0·014
	0·39 g.

% sterol = 17·23 %.

0·266 g. fat dissolved in 90 % alcohol and precipitated with 25 cc. of a 1 % solution of digitonin in 95 % alcohol gave 0·051 g. or allowing for solubility = 0·058 g.

$$\left.\begin{array}{l}\% \text{ unsaponifiable matter} = 39{\cdot}8\ \% \\ \% \text{ combined sterol} \quad = 12{\cdot}3\ \% \\ \% \text{ free sterol} \quad = 4{\cdot}9\ \%\end{array}\right\} 17{\cdot}2\ \%.$$

Yeast sterol very rapidly undergoes alteration; some decomposition appears to take place during the saponification process and it seems probable that the figures obtained for the sterol are too low. It is also probable that the solubilities of the yeast sterol and the cholesterol compounds with digitonin are not identical; the figures given can therefore only be regarded as approximate, and as representing the minimum amount of sterol in the sample.

Constituents of Yeast Fat.

The Fatty Acids.

Nägeli and Loew [1878] first investigated yeast fat and described its fatty acid as consisting mainly of oleic acid. Gérard and Darexy [1897] found in it butyric, palmitic and stearic acids. Hinsberg and Roos [1903] isolated from it three fatty acids, (1) an acid identical or isomeric with oleic, (2) an unsaturated acid having the formula $C_{12}H_{22}O_2$, and (3) a saturated pentadecoic acid to which they ascribed the formula $C_{15}H_{30}O_2$. These observers noted the ready oxidisability of the acids, in one instance the bulk of the acid having been converted to a neutral substance after standing for three weeks. The following year [1904] they succeeded in isolating and identifying a specimen of palmitic acid from the alleged pentadecoic acid.

Neville [1913], apparently in ignorance of the later work of Hinsberg and Roos, confirmed the presence of the acid $C_{15}H_{30}O_2$ which he was unable to separate into palmitic and a lower acid. He also isolated a substance melting at 77° which he regarded as arachidic acid; from a study of the oxidation products of the unsaturated acids he deduced the presence of an acid isomeric with linolic acid and of the C_{16} and C_{18} members of the oleic series.

We used as material for this part of the investigation, baker's yeast containing 2 % of fat calculated on the dry yeast. This had an iodine value of 121·2 and a saponification value of 187·5.

Preparation of the Methyl Esters.

Haller's method, *i.e.* heating with a 2 % solution of HCl in methyl alcohol, was first tried but is unsuitable when dealing with a very unsaturated fat such as that of yeast. The fat is little soluble in methyl alcohol: prolonged heating is necessary to bring about alcoholysis and during this time a heavy brown insoluble layer forms. These results resemble those obtained by Haller [1905] when working with linseed oil. Bull's method [1906] was therefore adopted.

100 g. of fat were dissolved in light petroleum and shaken with 26·18 cc. of a 4·25*N* solution of sodium methoxide in methyl alcohol. After standing two hours, water and light petroleum were added and the upper layer separated, washed with sodium carbonate solution and dried with $CaCl_2$. After distilling off the solvent, the methyl esters were twice distilled under 20 mm. pressure with a six-pear distilling column. The following fractions were obtained:

B.P.			
145–155°	...	...	1·1 g.
155–180°	...	...	0·2 g.
180–195°	...	...	2·3 g.
195–200°	...	...	9·0 g.
200–205°	...	...	0·5 g.
205–210° 210–215°	...	...	21·0 g.
215–220°	...	...	6·0 g.

There remained a considerable brown residue in the flask after the first distillation which decomposed on further heating.

Identification of Saturated Acids.

The fraction 195–200° partly solidified on standing; the solid ester was separated, pressed out, hydrolysed and the acid recrystallised from alcohol. After nine recrystallisations an acid melting at 61–62° was obtained, the melting point not being raised by further recrystallisation.

Analysis showed this to contain 75·00 % C and 12·28 % H. Calculated for palmitic acid 75·00 % C and 12·5 % H. The fraction boiling from 145 to 155° had an iodine value of 61; it was hydrolysed and the acid recrystallised, a solid acid separated which after recrystallisation melted unsharply at 38°. Methyl laurate boils at 148 under 18 mm. pressure; lauric acid melts at 43·6°. It seems probable therefore that this acid is lauric acid, but there was not sufficient material to confirm this further. Where only a small proportion of the C_{12} acid is present, it is difficult to remove the linoleic and oleic acids completely by distillation, unless large quantities of the acids are available. It may perhaps be regarded as confirmatory evidence that the melting point of the pentadecoic acid given by Hinsberg and Roos [1903] as 56° agrees well with that for a mixture of 20 % lauric and 80 % palmitic acid, 57·4° [Heintz, 1854]; the analytical figures they give also agree with those required by such a mixture (cp. Table I). A comparison of the results obtained by the various observers is shown in Table I.

Table I. Saturated fatty acid isolated from yeast fat.

Observer's name	M.P.	% C.	% H.	M.P. Methyl Ester
Hinsberg and Roos (1903)	56°	74·5	12·3	
„ „ (1904)	62°	74·85	12·67	
Neville (1913)	59°	74·59	12·38	26
Gérard and Darexy (1897)	60–61°			
MacLean and Thomas	61–62°	75·00	12·28	
Palmitic acid	62°	75·00	12·5	28
Pentadecoic acid	53°	74·38	12·39	
Mixture 20 % lauric and 80 % palmitic acids	57·4°	74·4	12·4	

The presence of palmitic acid is therefore definitely established and Neville's pentadecoic acid is probably a mixture of palmitic with a small amount of lauric acid.

In agreement with Neville's work, from the highest boiling fraction of methyl esters we isolated after hydrolysis a small amount of an acid melting at 77°: this was regarded by Neville as arachidic acid.

Identification of Unsaturated Acids.

	B.P.	Iodine value
Fraction of yeast fat methyl esters	200–205° (20 mm.)	96
" " "	205–215° "	130
" " "	215–220° "	123
Methyl oleate	212–213° (15 mm.)	85·8
Methyl linolate	207–208° "	172·7

It follows from the boiling points and iodine values of the distilled methyl esters that the bulk of the yeast fatty acids consists of a mixture of linoleic and oleic acids. Bromination of the free acids gave a mixture of bromides totally soluble in ether. From this, a solid bromide was obtained, insoluble in light petroleum, which after being twice recrystallised from alcohol melted at 113–114°. It was therefore identified as tetrabromo-linoleic acid. The solution in light petroleum contained liquid dibromo-oleic acid.

Hinsberg and Roos [1903] isolated a small amount of an acid which from its analytical numbers they regarded as an unsaturated dodecenic acid. It was separated from the ether-soluble lead salts, which, as has been pointed out by Lewkowitsch [1913], contain also small amounts of the C_{12} and lower saturated fatty acids if these are also present in the mixture. The iodine value of the acid was not determined and the evidence for the existence of a naturally occurring dodecenic acid cannot yet be regarded as convincing. One of us [Smedley, 1912] noted on distilling the esters of the butter fatty acids a rise in the iodine value of the fraction corresponding to the C_{12} acids and suggested as a possible explanation the presence of a dodecenic acid in butter fat. Crowther and Hynd [1917] distilled a mixture of oleic acid with a mixture of saturated fatty acids and found under these circumstances a curve similar to that obtained with the butter fat esters, giving a perceptible increase of iodine value for the C_{12} fraction. The presence of an unsaturated C_{12} acid in yeast, while therefore not definitely excluded, must be regarded as doubtful.

Lecithin.

The presence of lecithin in yeast fat was first demonstrated by Sedlmayer [1903] who described it as dipalmitocholine-glycerophosphoric acid. He obtained 4 % crude lecithin calculated on the dried yeast, and estimated the amount of pure lecithin as 2 %. Since the whole of the ether-soluble fraction, consisting mainly of sterol and fat, was regarded as the crude lecithin, these figures are too high. We found that the fraction precipitable from ether or alcohol solution by the addition of acetone gave an average value of 8·5 % of the total fat or about 0·17 of the weight of dried yeast.

The Sterol of Yeast.

Gérard [1895] isolated from yeast a plant cholesterol melting at 135–136° and regarded it as belonging to the same group as the ergosterol obtained by Tanret [1889] from ergot. The latter substance however melted at 154°. Gérard regards ergosterol as typical of a group of sterols characteristic of the cryptogams and differentiated by their reaction with $CHCl_3$ and concentrated H_2SO_4 from the phytosterols and cholesterols characteristic of the higher plants and animals respectively. The latter substances when concentrated sulphuric acid is added to their chloroform solutions, impart a red colour to the chloroform solution, the acid layer becoming fluorescent. With ergosterol however under similar conditions the chloroform solution remains colourless or shows a slight green fluorescence, the acid taking on a deep red colour.

Hinsberg and Roos [1903] isolated two sterols one melting at 159° and one at 148–149°; the melting point of the latter they were unable to raise by recrystallisation. They state that the sterol of M.P. 159° gives a red colour with chloroform and concentrated acid but do not say which layer contains the red colour. They found that their sterol was not identical with the caulosterol (M.P. 158–159°) isolated by Barbieri and Schulz [1882]. Analysis of the sterol after drying over sulphuric acid gave them results agreeing with the formula $C_{26}H_{44}O$. Neville [1913] who next investigated this substance, isolated only a sterol melting at 148–149°.

Preparation of Yeast Sterol.

The fat from baker's yeast and from the brewer's yeast supplied by Dr Thaysen, extracted as described above, was saponified with alcoholic potash, the solution neutralised and extracted with ether, the ether-soluble fraction being recrystallised from alcohol and finally from ether; crystals of the sterol also separated on allowing the fat to stand in the cold.

By repeated recrystallisation of the sterol from ether, we obtained a substance melting at 154°, which appears to be identical with the ergosterol isolated by Tanret from ergot [1889, 1908].

Our yeast sterol when first isolated melted at 135–136° and many recrystallisations were necessary to raise the melting point to 154°. The melting point of ergosterol is given by Tanret as 154° "à l'état brut" and 165° on the Maquenne block. Solutions of yeast sterol become yellow and decompose slightly on heating or even on standing at ordinary temperature, a yellow oily substance being formed. It is therefore difficult to obtain in the pure state by crystallisation, and the discrepancies in the melting point between the results of the specimens of the yeast sterol described by different observers are probably due to this cause. On adding concentrated sulphuric acid to a solution of yeast sterol in chloroform, the acid layer is coloured red. The close agreement in melting point and specific

Table II. Properties of sterols isolated from Cryptogams.

Name	Formula ascribed	Source	% C. (anhydrous)	% H (anhydrous)	Melting-point	Rotation in $CHCl_3$ $[\alpha]_D$	Rotation in ether $[\alpha]_D$	M.P. acetate	Rotation acetate $[\alpha]_D$	Observer
Ergosterol	$C_{28}H_{40}O$, H_2O	Ergot	84·6 85·0 84·64	11·06 11·20 11·25	154°	−114°		169–175°	−80°	Tanret [1889]
,,	$C_{27}H_{42}O$, H_2O	,,			154° à l'état brut 165° sur le Maquenne bloc	−126°	−105	180·5°	−91·8°	,, [1908]
,,	$C_{24}H_{40}O$, H_2O	,,			150°		−89·5 at 15°	165		Ottolenghi [1906]
Yeast sterol		Yeast (brewery)			135–136°	−105°				Gérard [1895]
,,	$C_{26}H_{44}O$	,, ,,	83·35	11·9	(*a*) 159° (*b*) 148–149°					Hinsberg and Roos [1903]
,,		,, ,,			156–157°					Meisenheimer [1915]
,,		Yeast			145–147°		−75·5°[1]			Neville [1913]
,,		Yeast (*a*) Baker's (*b*) Brewery			154° 154°	−117° at 18°		170·5	−87·3°	MacLean and Thomas
			(not dehydrated)							
Mycosterol	$C_{30}H_{48}O_2$	*Collybia shiitake*	82·00	10·81	159–160°	−129·4°		169°		Ikeguchi [1919]
,,	,,	*Armillaria edodes*	81·87	11·35	159–160°	−129·2°				,, ,,
,,	,,	*Hudnum asparatum*	82·02	10·95	159–160°	−129·5°				,, ,,
Fungisterol	$C_{25}H_{40}O$, H_2O	Ergot			144° sur le Maquenne bloc	−22·4°	−12·9°	158·5°	−15·9° (ether)	Tanret [1908]

[1] Solvent not stated.

rotation, of both yeast sterol and its acetate with the corresponding compounds of ergosterol is shown in Table II. Tanret showed that in ergot the ergosterol is accompanied by a sterol of formula $C_{25}H_{40}O$, H_2O, M.P. 144° (determined on the Maquenne block) to which he gave the name of fungisterol. It is possible that this is also present in yeast but we were unable to detect it.

In one specimen of yeast obtained from a local brewery no ergosterol could be detected but from the unsaponifiable matter a sterol melting at 97–98° and giving a specific rotation of + 10·1° in chloroform solution was isolated. The presence of a positive rotation and the low melting point suggest a resemblance to coprosterol (M.P. 99° and specific rotation + 24°) which is obtained by reduction of cholesterol in the intestines. It is possible that in this particular specimen reduction of the ergosterol had taken place giving a coprosterol-like compound. We hope to be able to obtain further information on this point.

The Iodine Value of the Sterols.

As stated above in the discussion of the iodine value of yeast fat, very high iodine values were obtained on treating yeast sterol with Wijs' reagent. The fact that the iodine value of cholesterol when determined by Wijs' method varies with the time of the reaction and with other conditions has been noted by Werner [1911] and by Lewkowitsch [1913], who found iodine values up to 145.

We examined the iodine value of cholesterol and of yeast sterol by the methods both of Hübl and of Wijs. With Wijs' solution the reaction is not confined to addition of halogen to the ethylene linkages and high values result. The acetates of the sterols behave similarly so that the alcohol group does not appear to be concerned in these changes. The presence of an appreciable quantity of sterols in any fat leads therefore to an abnormal value when determined by Wijs' reagent, and in such cases the iodine value cannot be taken as a measure of the number of ethylene linkages present in the molecule.

With Hübl's reagent the action with the sterol proceeds slowly and gradually, and falls off very much after 24 hours. Lewkowitsch found for the iodine value of cholesterol by Hübl's method the values 68·09 and 67·43. Theory for one unsaturated linkage in the molecule requires 62·8 corresponding to the formula $C_{27}H_{46}O$, H_2O. We found values of from 65 to 75 which correspond approximately with this theoretical requirement. Werner found by Hübl's method for phytosterol values from 41 to 76, according to the time of the reaction; after half-an-hour by Wijs' method 135.

We then tested a sample of brassicasterol supplied to us by Professor Windaus and found for this with Hübl's reagent an iodine value of 118; theory for two double bonds demands 122. Windaus and Welsch [1909] have shown that brassicasteryl acetate adds on four atoms of bromine and contains

therefore two double bonds, a result with which the Hübl iodine value is in good agreement. Yeast sterol and its acetate under similar conditions give iodine values corresponding to the presence of three double bonds in the molecule. This result is in agreement with the formula ascribed to ergosterol by Tanret, $C_{27}H_{42}O, H_2O$, a formula differing from that of cholesterol only in that it contains four hydrogen atoms less.

The evidence so far available (see Table III) therefore indicates that yeast sterol differs from the cholesterols and phytosterols yet described in that it contains three double bonds in the molecule.

Table III. Iodine Values of Sterols.

Substance and formula	I value (Wijs)	Time of reaction	I value (Hübl)	Time of reaction
Cholesterol $C_{27}H_{46}O, H_2O$		hrs.		hrs.
[I value calc. if one double bond is present = 62·8]	141·6	6	58·65	6
	152·7	18	70·21	18
	165·1	22·5	64·88	22·5
	165·8	22·5		
	153·2	24	73·2	24
	158·4	25		
	165·4	48	71·8	48
Cholesteryl acetate				
[I value calc. if one double bond is present = 59·8]	120·2	6	27·94	6
	127·8	18	48·26	18
	131·1	24	49·8	20
	144·1	48	52·1	24
			62·6	48
Brassicasterol $C_{28}H_{46}O, H_2O$ [I value calc. if two double bonds are present = 122·0]			118	22·5
Sterol from yeast, $C_{27}H_{42}O, H_2O$ [I value calc. if three double bonds are present = 190·5]	337·8	23	177·1	21
Steryl Acetate from yeast [I value calc. if three double bonds are present = 179·1]	245·2	23	175·1	

Probable identity of Mycosterol with Ergosterol.

Recently Ikeguchi [1919] isolated a sterol from various fungi, *Armillaria edodes*, *Collybia shiitake*, *Hydnum asparatum* and *Lycoperdon gemmatum*, to which he gave the name of mycosterol. Its properties closely resemble those of ergosterol. It gives similar colour reactions when treated with chloroform and concentrated sulphuric acid, the red colour is in the acid layer, and its melting point, rotation and the melting point of its acetate are all in close agreement with those of ergosterol (see Table II). The difference in the analytical numbers may be due to the fact that Ikeguchi apparently analysed the crystals after recrystallising them from alcohol, when they contain one molecule of water of crystallisation, whereas other observers state that they dried to a constant weight over sulphuric acid.

Ikeguchi's numbers agree fairly with those required for the hydrated sterol. The percentage of carbon is low but this is possibly due to slight loss of water of crystallisation. Ikeguchi shows that only one acetyl group is introduced into the molecule on acetylation, and that a carbonyl group is

not present. He does not however consider the possibility of the second oxygen atom being present as water of crystallisation.

The only property of mycosterol which is not in agreement with our observations on yeast sterol is its behaviour on bromination. When bromine in acetic acid was added to an ether solution of mycosterol the crystals recovered agreed in melting point and in colour reactions with the original substance, and the conclusion is drawn that no double bond is present in the molecule. When a similar experiment was performed with yeast sterol, the bromine solution was rapidly decolorised and a black oily product was obtained on evaporating the solution, which solidified on standing, but we had not sufficient material to isolate a pure substance from this. In view however of the close agreement of mycosterol with ergosterol, further investigation of its behaviour on bromination seems desirable.

We are at present continuing our investigations on the constitution of yeast sterol.

Summary and Conclusions.

1. Palmitic, oleic and linoleic acids have now been identified with certainty in yeast fat.

2. The pentadecoic acid previously described consists of a mixture of palmitic and lauric acids. Evidence for the presence of lauric acid has been obtained.

3. The presence of an acid melting at 77° has been confirmed, the melting point of which agrees with that of arachidic acid.

4. No confirmation of the presence of the dodecenic acid described by Hinsberg and Roos was obtained and the possibility of this being a mixture of lauric with oleic and linoleic acids has not yet been excluded.

5. A sterol is present partly in the free state and partly as fatty acid esters; this may constitute 20 % of the total yeast fat.

6. The sterol present in yeast appears to be identical with the ergosterol isolated from ergot by Tanret.

7. The variation in melting point described by different observers seems to be due to differences in the purity of the substance, but no conclusive evidence of the presence of a second sterol in yeast has yet been obtained.

8. It seems probable that the mycosterol isolated by Ikeguchi from certain fungi is also identical with ergosterol and that ergosterol is characteristic of the whole group of cryptogams just as cholesterol is of the animal and phytosterol is of the higher plant kingdom.

9. Yeast sterol is differentiated from the sterols of the higher plants and animals by the presence of three double bonds in its molecule.

Our thanks are due to Dr Thaysen who provided us with some of the material used in this investigation. The work was carried out for the Food Investigation Board of the Department of Scientific and Industrial Research and we gratefully acknowledge the grants provided for this purpose.

REFERENCES.

Barbieri and Schulz (1882). *J. pr. Chem.* (2), **25**, 159.
Bull (1906). *Ber.*, **39**, 3570.
Crowther and Hynd (1917). *Biochem. J.*, **11**, 139.
Gérard (1895). *J. Pharm. Chim.* (6), **1**, 601.
—— and Darexy (1897). *J. Pharm. Chim.* (6), **5**, 275.
Haller (1905). *Compt. Rend.*, **143**, 657.
Heintz (1854). *Annalen*, **92**, 295.
Hinsberg and Roos (1903). *Zeitsch. physiol. Chem.*, **38**, 1.
—— (1904). *Zeitsch. physiol. Chem.*, **42**, 189.
Ikeguchi (1919). *J. Biol. Chem.*, **40**, 175.
Lewkowitsch (1913). *Chemical Technology of Oils, Fats and Waxes* (Macmillan).
Meisenheimer (1915). *Wochenschr. f. Brauerei*, **32**, 325.
Nägeli and Loew (1878). *J. pr. Chem.*, **17**, 403.
Neville (1913). *Biochem. J.*, **7**, 347.
Ottolenghi (1906). *Chem. Centr.*, 1906, **1**, 541.
Sedlmayer (1903). *Zeitsch. f. das ges. Brauwesen*, **26**, 381.
Smedley (1912). *Biochem. J.*, **6**, 451.
Tanret (1889). *Compt. Rend.*, **108**, 98.
—— (1908). *Compt. Rend.*, **147**, 75.
Werner (1911). *Inaug diss.*, Berlin.
Windaus (1909). *Ber.*, **42**, 238.
—— and Welsch (1909). *Ber.*, **42**, 612.

XLI. THE EXTRACTION OF THE FAT-SOLUBLE FACTOR OF CABBAGE AND CARROT BY SOLVENTS.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(*Received April 29th, 1920.*)

The study of the fat-soluble accessory factor in fats has so far not contributed anything very definite to our knowledge of the chemistry of this principle. Observations so far made on active fats favour the view that the activity is not due to the fat itself but to some substance or substances which are in association with it. The fat-soluble factor is however not easily separated from it by differential solvents. All other conceivable methods of fractionation are mostly of a drastic nature not suitable for the manipulation of a physiologically active principle of doubtful stability.

It is now well established that some vegetables form a rich source for a substance physiologically resembling the fat-soluble accessory factor in fats but as in the case of the fats little is known of its chemical character. We are even yet unaware whether it is present in the vegetable cell in solution or in suspension. According to McCollum, Simmonds and Pitz [1916, 1] the fat-soluble factor is not extracted from plants with the fats by ether, chloroform, benzene or acetone. Hot alcohol however according to these authors [1916, 2] removes the factor from maize kernel.

This communication describes some experiments in which active fractions were obtained from fresh vegetables by extraction with absolute alcohol and eventually with ether.

Originally it was intended to study the fat-soluble factor in cabbage but owing to some technical difficulties the investigation was extended to carrots. It has been found by various workers that cabbage is rich in the fat-soluble factor. Preliminary experiments carried out in connection with this research have shown that small quantities of this vegetable in fresh condition were sufficient to promote growth in rats fed on a diet lacking the factor. The aim was to treat the minced cabbage at first with a solvent which would fulfil the dual function of disorganising the plant cells and of extracting the accessory factor. Absolute alcohol was chosen and found to be suitable for the purpose. However the extract thus obtained was not relished by the rats who mostly either refused the food containing it or consumed insufficient

of it. It was therefore found necessary to apply this method of extraction to another vegetable, namely carrots, the extract of which was likely to prove more palatable to the animals. This vegetable although not very rich in the fat-soluble accessory factor was shown by Denton and Kohlman [1918] and Steenbock and Gross [1919] to contain appreciable quantities of it. As anticipated it was found possible to extract by means of alcohol from the carrots an active fraction which the animals took well. This extract afforded the opportunity of further fractionation, and it was found that ether extracted the active principle from it leaving the best part of the extraneous matter behind. As the carrot also contains the antineuritic and antiscorbutic factors it was conjectured from theoretical considerations that the alcoholic extraction would also remove these two factors. Experiments instituted with that purpose have indeed shown that the extract contained the antineuritic and to a smaller extent the antiscorbutic factor. These two principles were however not present in the extract in such high proportions as the fat-soluble factor.

Experimental.

Fresh green cabbage was thoroughly minced and triturated with sand. Absolute alcohol was then added (500 cc. of absolute alcohol for every 100 g. of cabbage) and the mixture allowed to digest in a cool dark place for about 12–18 hours. It was then filtered through a fluted filter and the residue on the filter paper pressed out and filtered. The combined filtrates were green and transparent and possessed the usual appearance of an alcoholic solution of chlorophyll. The alcoholic extract was then evaporated *in vacuo* at 35°. As the solution became concentrated chlorophyll separated out leaving behind a light brown liquid. The addition of alcohol to this solution which was practically free from alcohol produced a small white precipitate which consisted most probably of protein. On further concentration a greenish brown sweet syrup containing reducing sugars was obtained. This syrup possessed the characteristic taste of boiled cabbage. An equivalent of 25 g. of fresh cabbage was added daily to a diet which was complete in every respect but which lacked the fat-soluble factor. The composition of this BC diet was described in a previous communication [Zilva, 1919]. Of the various rats fed on this diet only few consumed the food well enough to demonstrate the growth-promoting power of the extract. Fig. 1 gives the weight curves of two representative rats. The male animal consumed the best part of his food, on the average about 18 g. per day, with the result that it grew well and reached a satisfactory weight within the 11 weeks during which it was kept on the diet. The female animal consumed only about 10–12 g. of the food per day. Such an amount is hardly quantitatively adequate to promote normal growth even if the diet were qualitatively well balanced. It will be seen that although the animal grew, the rate of growth was below the normal. The experiments with the cabbage extract, however, served as a

preliminary indication and demonstrated that it was possible to extract the fat-soluble factor by the method described.

In order to obviate the irregularity in the food intake of the rats carrots were next employed as a source for the accessory factor. These vegetables were treated in the same way as the cabbage. The filtrate obtained in this case was brilliantly clear and yellow in colour. On concentration *in vacuo* at 35° a very sweet and pleasant-tasting syrup was obtained. The syrup could be dissolved in water producing a turbid reddish yellow solution. An equivalent of 25 g. of fresh carrots was given to the animals in their daily diet at first. This preparation the animals consumed very well and as will be seen from Figs. 2 and 3 they developed well on it. Fig. 2 represents the

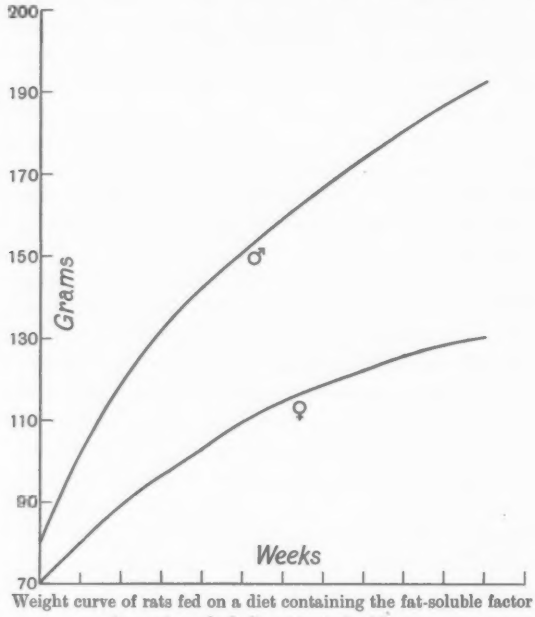


Fig. 1. Weight curve of rats fed on a diet containing the fat-soluble factor in the form of an alcoholic extract of cabbage.

weight curves of three animals which received the extract after they had subsisted for three weeks on a diet lacking the fat-soluble factor. They immediately resumed normal growth instead of ceasing to grow as was the case with the control animal. Fig. 3 shows how, on administration of an equivalent of 25 g. of fresh carrots to an animal which had existed on a diet free from the fat-soluble accessory factor for ten weeks and which was rapidly losing in weight, normal growth was induced.

It was next desirable to ascertain the minimum dose of the extract that would induce normal growth. Doses equivalent to 15 g., 5 g., 1 g. of fresh

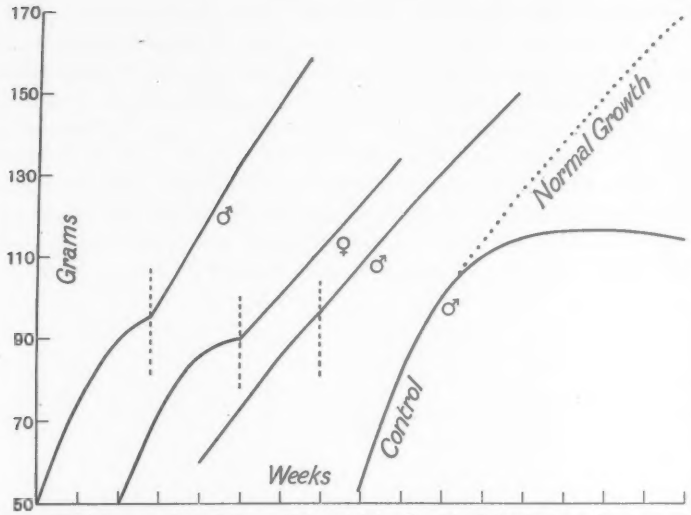


Fig. 2. Weight curve of rats fed on a diet containing the fat-soluble factor in the form of an alcoholic extract from carrots. The perpendicular dotted lines denote the commencement of the treatment.

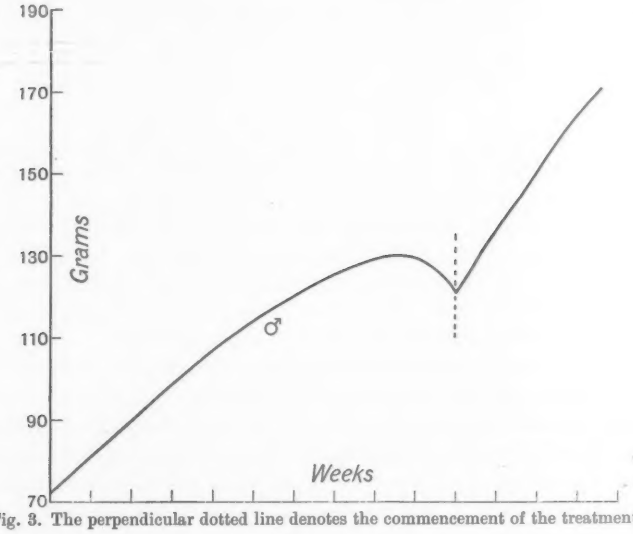


Fig. 3. The perpendicular dotted line denotes the commencement of the treatment.

carrots were given to three rats. In every case growth was induced (Fig. 4). The addition of the equivalent of 15 g. of carrots produced almost normal growth. The extracts from 5 g. and 1 g. produced less intense growth. An animal which had ceased growing for several months owing to the deficiency of the fat-soluble factor also resumed growth on receiving a dose equivalent to 1 g. of carrot. The growth curve in this case commenced flattening out after about ten days. The diet containing the 1 g. was consumed entirely. About 80 %–90 % of the other two doses was consumed daily by the rats. It may be pointed out in conjunction with the above figures that Steenbock and Gross [1919] found that 5 % of dried carrot was insufficient to produce normal growth while a diet containing 15 % sufficed for the purpose.

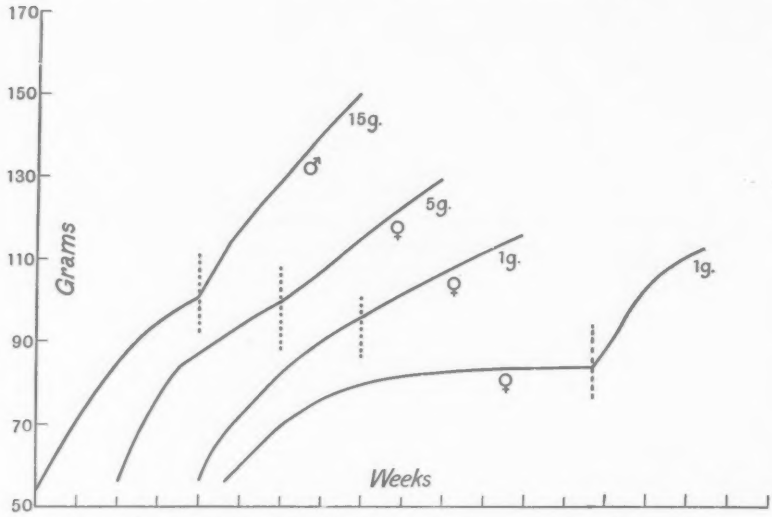


Fig. 4. The perpendicular dotted lines denote the commencement of the treatment.

Absolute alcohol extracts in the form of the syrup about 7·5 % of dry matter from fresh carrots which comprises about one-half of the total solids. On drying the syrup *in vacuo* at a low temperature and keeping the residue in a desiccator it was found that after a fortnight an equivalent of 25 g. of carrots was active both in inducing good growth and in curing xerophthalmia in rats. Any further particulars of the keeping properties of the carrot extract in dry condition have not yet been worked out.

The antineuritic factor was tested out on rats. Equivalents of 25 g. per day were added to an AC diet [Zilva, 1919]. It will be seen from Fig. 5 that this dose was sufficient to induce development a little below the normal rate. As these animals only consumed about 70 % of their daily diet it may be assumed that the dose was about the minimum necessary for normal growth.

Fig. 5 also shows the weight curve of a rat which was deteriorating on account of an antineuritic deficiency but which on receiving the extract equivalent to 25 g. of fresh carrots resumed growth.

The tests for the antiscorbutic factor showed that the content of this principle in the carrot extract was very low. Three guinea-pigs kept on a diet of oats and bran and a daily ration of 40 cc. of autoclaved whole milk received after the tenth day a daily dose of the extract equivalent to 25 g., 10 g. and 5 g. of fresh carrots respectively. The animals which received the 10 g. and 5 g. doses developed scurvy, declined in weight and eventually died in

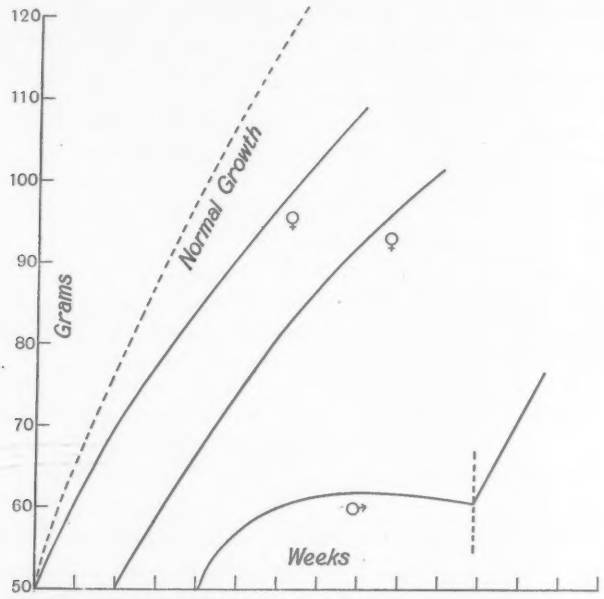


Fig. 5. The perpendicular dotted line denotes the commencement of the treatment.

the ordinary way as they would have done on a purely scorbutic diet (Fig. 6). No delay in the onset of the symptoms or in the fatal termination could be recorded. The guinea-pig which received daily the extract from 25 g. of fresh carrots showed a decided delay in the onset of the scorbutic symptoms. Unfortunately this animal succumbed to some other complaint on the 23rd day. At the post mortem examination mild signs of scurvy were observed which were not acute enough to be responsible for the death of the animal. The extract from 25 g. of carrots contains therefore enough antiscorbutic to delay the onset of the disease[1].

[1] [Note added on 9th of June.] A daily administration of an alcoholic extract equivalent to 50 g. of fresh carrots protected a guinea-pig subsisting on a scorbutic diet for 42 days.

The alcoholic extract from carrots was next concentrated until all the alcohol was driven off and the aqueous solution became concentrated. This solution was extracted by shaking it with ether in a separating funnel. The ethereal solution at first was yellow but on repeated extraction no more colouring matter could be removed from the solution which nevertheless still retained a fairly intense yellow colour. A solution equivalent to 250 g. of fresh carrots extracted with ether as described yielded a residue weighing 0·4935 g. The residue, which was dark brown, on drying for two hours at 110° remained of oily consistency. On extracting it with sodium carbonate and acidifying the solution no precipitate was obtained which would indicate that it did not contain any free higher fatty acids. The residue was next saponified by heating with alcoholic potash under a reflux condenser for two hours, the alcohol driven off, and the aqueous solution filtered. On adding acid to the filtrate a solid substance resembling the higher fatty acids separated out and gradually accumulated on the surface of the liquid. There is little doubt then that the ethereal extract contained some oil.

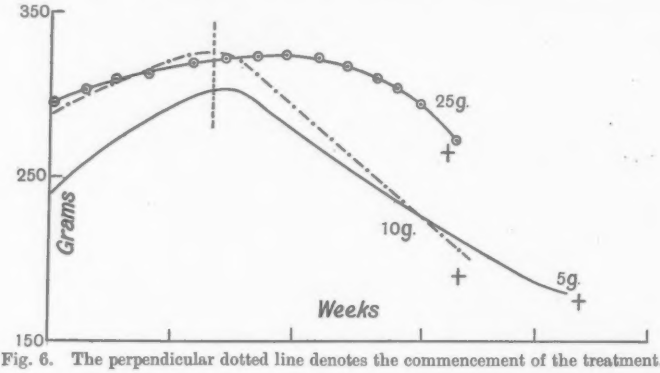


Fig. 6. The perpendicular dotted line denotes the commencement of the treatment.

In order to test the activity of this fraction autoclaved olive oil was added to the ethereal solution and the ether evaporated. A highly coloured and opaque oil was left behind. Both the colour and the opacity were dependent on the strength of the extract. Through the medium of the oil the ethereal extract, equivalent to 25 g. of carrots, was incorporated in the daily diet which was free from the fat-soluble factor and as will be seen from Fig. 7, which represents the weight curve of two rats treated with this fraction, growth was promoted immediately the treatment was commenced.

While these experiments were in progress Osborne and Mendel [1920] published a short note in which they state that they have extracted with ether from spinach leaves and young clover dried at 60° an oily residue which was active in small quantities. Although they have obtained their fraction from different plants and directly from the dried vegetable their work corroborates the fact that the fat-soluble factor in plants is soluble in ether. Further

experiments with various solvents carried out preferably on the alcoholic fraction ought to throw some light on the subject whether the activity of the ethereal fraction is due to the small amount of the oil or to some other substance associated with it.

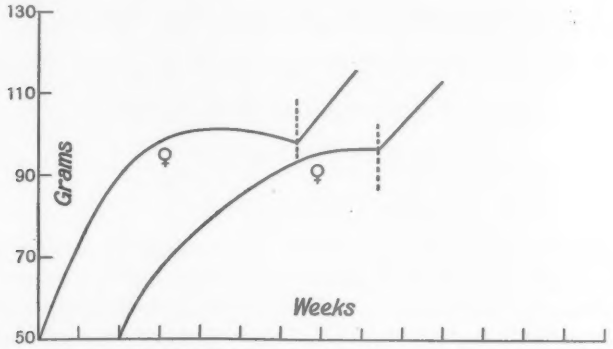


Fig. 7. The perpendicular dotted lines denote the commencement of the treatment.

Summary.

1. Alcohol extracts the fat-soluble factor from cabbage and carrots.

2. The alcoholic extract equivalent to 10–12 g. of fresh carrots given daily is sufficient to promote normal growth in rats subsisting on a diet lacking the fat-soluble factor.

3. The alcoholic extract from carrots also contains the antineuritic and to a smaller extent the antiscorbutic factors.

4. An ethereal extract from the alcoholic fraction equivalent to 25 g. of fresh carrots has been found to promote recovery and renew growth in rats declining in weight on account of a fat-soluble factor deficiency.

Part of the expense of this research was defrayed from a grant made by the Medical Research Council, to whom my thanks are due.

References.

Denton and Kohlman (1918). *J. Biol. Chem.*, **36**, 249.
McCollum, Simmonds and Pitz (1916, 1). *Amer. J. Physiol.*, 1916, **41**, 333.
—— (1916, 2). *J. Biol. Chem.*, **28**, 153.
Osborne and Mendel (1920). *J. Biol. Chem.*, **41**. Proc. Amer. Soc. Biol. Chemists vii.
Steenbock and Gross (1919). *J. Biol. Chem.*, **40**, 501.
Zilva (1919). *Biochem. J.*, **13**, 164.

XLII. A CONTRIBUTION TO THE STUDY OF KERATOMALACIA AMONG RATS.

By MARJORY STEPHENSON and ANNE BARBARA CLARK.

From the Biochemical Laboratory, Cambridge.

(*Received May 11th, 1920.*)

(With Plates IX and X.)

The association of acute mal-nutrition amongst children with a purulent condition of the eyes has long been familiar in medical practice. Mori [1904] associated a condition which he called "xerosis of the conjunctiva and keratomalacia," frequent amongst famine-stricken children, with fat starvation, and noted the curative effect of chicken liver and fish oils. He also observed that the disease occurred among populations using vegetable oils but was absent in sea-board districts where fish oils were used.

More recently Bloch [1917] associated an outbreak of corneal ulceration amongst children with a diet of highly separated milk, and noted the curative effect of whole milk and cod liver oil.

In 1917 McCollum and Simmonds [1917, 1], working with rats on deficiency diets, definitely associated a purulent condition of the eye—which they termed "xerophthalmia"—with the absence from the diet of the fat-soluble vitamine. In the above and a subsequent paper [McCollum and Simmonds, 1917, 2], these authors claim that xerophthalmia is an undoubted deficiency disease. They write: "There are then two deficiency diseases in the sense in which Funk employed this term. One of these is polyneuritis.... The other we believe is the syndrome described above in which the two most prominent features are emaciation and xerophthalmia" [1917, 1].

In order to make good the claim of keratomalacia, a term which we prefer to xerophthalmia, to rank as a deficiency disease in the strict sense of the term (due, in this case to the absence of the fat-soluble vitamine) it is necessary to establish the following points in respect of it:

1. That the determining cause is not bacterial infection.

2. That in one species, at least, the symptoms invariably result from a prolonged course of diet deficient in the fat-soluble factor.

3. That identical symptoms never occur as a result of any form of mal-nutrition in which the fat-soluble factor is adequately supplied in the diet.

4. That the symptoms invariably disappear when the factor is replaced in the diet (except in cases where death occurs before any general improvement can be manifested).

5. That the symptoms are not susceptible of cure by any treatment other than the supply of the fat-soluble factor.

As a contribution to this subject we have attempted to deal with Nos. 1, 2 and 4 of the above points, Nos. 3 and 5 falling outside the scope of our investigation.

The material dealt with in this communication, therefore, falls under two main headings.

A. Dietetic and Statistical.

B. (1) Histological. (2) Bacteriological.

A. DIETETIC AND STATISTICAL.

a. Incidence of the disease.

As has already been noted (p. 502) in order to weigh the claims of keratomalacia among rats to rank as a deficiency disease it is necessary to study statistically the cases which occur when these animals are placed on a diet deficient in the fat-soluble factor. For this purpose the results of eight experiments, including in all 46 rats, are here given. The rats were all young (between 40 and 50 g. in weight) at the beginning of the experiment. The diet consisted of the usual combination of purified caseinogen, starch, sugar, and vegetable fat (usually palm kernel oil), and McCollum's salt mixture [McCollum and Simmonds, 1917, 1], to which were added traces of sodium fluoride, potassium iodide, and manganese sulphate. The water-soluble factor was supplied by a fat-free alcoholic extract of yeast and the anti-scurvy factor by 0·5 cc. of lemon juice per rat per day. In Table I the results of these experiments are recorded; Table II summarises the results of the eight experiments. The duration of the experiment is divided into periods of ten days; in each of these periods are recorded:

1. The total number of deaths occurring in the period.
2. The number of deaths with eye disease occurring in the period.
3. The total number of survivors at the end of the period.
4. The survivors with eye disease at the end of the period.
5. The cases of eye disease occurring during the period.

Fig. 1 represents the deaths occurring in the successive periods of the experiment; the blackened portion indicates the number of those deaths preceded by eye disease. It is seen from this that no deaths occurring before the 50th day were preceded by eye disease; after that period an increasing proportion of deaths were preceded by symptoms of keratomalacia, but *in no period* are *all* the deaths so preceded.

Fig. 2 represents the number of rats surviving at the end of any one period; the blackened portion represents those survivors having eye disease. It is here seen that the incidence of the disease increased with the duration of the experiment, but that at *no* period are *all* the surviving rats afflicted with the symptoms.

Table I.

No. of rats on experiment		Day of experiment on which deaths occurred	Day of experiment on which eye disease first noted	No. of rats which had eye disease	No. of rats which had no eye disease	No. of survivors
I.	5	29	No eye disease	1	4	0
		52	" "			
		79	" "			
		80	64th day			
		80	No eye disease			
II.	5	11	" "	2	3	0
		11	" "			
		40	" "			
		85	77th day			
		86	77th day			
III.	5	65	61st day	4	1	0
		65	61st day			
		65	No eye disease			
		80	77th day			
		86	77th day			
IV.	5	15	No eye disease	2	3	0
		15	" "			
		31	" "			
		82	77th day			
		86	56th day			
V.	6	47	No eye disease	4	2	0
		54	50th day			
		57	57th day			
		78	No eye disease			
		79	47th day			
		104	82nd day			
VI.	5	26	No eye disease	0	5	0
		38	" "			
		38	" "			
		38	" "			
		38	" "			
VII.	9	15	" "	0	9	0
		27	" "			
		30	" "			
		30	" "			
		31	" "			
		31	" "			
		31	" "			
		41	" "			
		43	" "			
VIII.	6	26	" "	0	6	1
		26	" "			
		56	" "			
		64	" "			
		88 (killed)	" "			
Total	46	—	—	13	33	1

Table II. Summary of eight experiments.

	Day											
	0 to 9	10 to 19	20 to 29	30 to 39	40 to 49	50 to 59	60 to 69	70 to 79	80 to 89	90 to 99	100 to 109	110 to 119
Total deaths	0	5	5	10	4	4	4	3	9	0	1	0
Deaths with eye disease ...	0	0	0	0	0	2	2	1	7	0	1	0
Total survivors (end of period)	46	41	36	26	22	18	14	11	2	2	1	1
Survivors with eye disease (end of period)	—	—	—	—	1	2	3	7	1	1	0	0
Cases of eye disease occurring	0	0	0	0	1	3	3	5	1	0	0	0

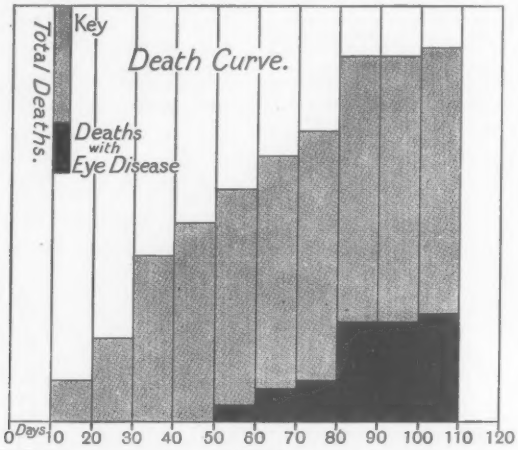


Fig. 1.

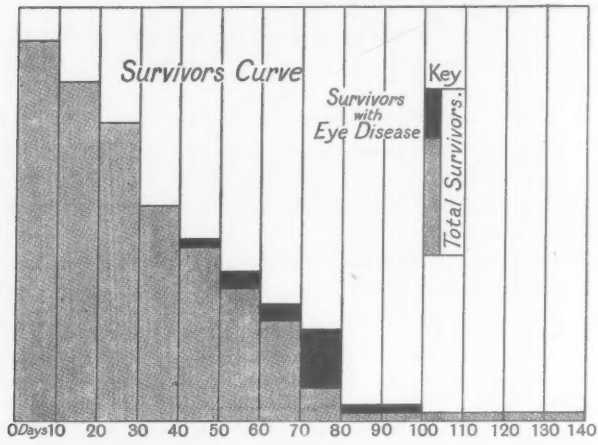


Fig 2.

These facts taken in conjunction suggest: (1) That a diet deficient in the fat-soluble factor, whilst strongly predisposing the subject to eye disease, does not invariably result in it[1]. (2) That the occurrence of the disease is not coincident with the cessation of growth, but begins at a later stage.

β. Cure of the Eye Disease.

In ten cases of eye disease (distinct from any appearing in the previous tables) cure was attempted; the results are tabulated below (Table III).

Table III.

Day of experiment on which disease was first noted	Day of experiment on which cure was attempted (*i.e.* fat-soluble factor added to the diet)	Day of experiment on which cure was complete	Day of experiment on which rat died
72nd	82nd	86th	96th
54th	82nd	93rd	106th
72nd	82nd	107th	survived
50th	54th	60th	62nd
54th	54th	67th	101st
50th	54th	60th	survived
57th	60th	64th	,,
37th	39th	46th	,,
37th	39th	46th	,,
36th	39th	43rd	,,

These experiments were made in connection with a separate investigation; the material carrying the fat-soluble factor was a light petroleum extract of dried carrot which was incorporated with the palm kernel oil. This material was shown in control experiments (including 21 rats in all) to be carrying the fat-soluble factor.

It is seen that *in every case* the eye symptoms cleared up though the time taken to effect the cure varied considerably; it is noteworthy however that four out of the ten rats failed to survive.

Thus of 46 rats on a diet deficient in the fat-soluble factor 96 % failed to survive 90 days, and 28 % contracted eye disease. Of ten rats which had contracted disease and were given the fat-soluble factor 100 % were cured of the disease, but only 60 % restored to health, 40 % dying after the eye symptoms had cleared up.

From these two sets of experiments (α and β) an apparent paradox emerges; absence of the fat-soluble factor almost invariably causes death (preceded by

[1] It is our opinion that the non-appearance of "xerophthalmia" on diets deficient in the fat-soluble factor recorded from this laboratory [Bulley, 1919] was due to the fact that for a period during the war the caseinogen used for the experimental animals was insufficiently extracted with alcohol. We have since found that a small amount of lipoid material, somewhat rich in the factor, was present in this caseinogen and in our belief protected the rats from eye disease. This, and the comparative rarity of the disease in the human subject, suggests that keratomalacia only appears when the diet is very highly deficient in the fat-soluble factor.

cessation of growth) and *sometimes* produces eye disease; replacement of the fat-soluble factor *invariably* cures the eye disease but sometimes (on the same rats) fails to restore growth and save life.

The first explanation of this apparent paradox that suggests itself is that the concentration of the fat-soluble factor in the tissues necessary to protect the eye is smaller than that necessary to ensure normal growth; this would account both for the later incidence of the disease and its more certain cure. It does not however afford a satisfactory explanation of all the facts. Supposing the concentration of the fat-soluble vitamine (in the tissues) necessary to life be x, while that necessary to protect from eye disease be y. In the case of the rats dying before they contract eye disease x is greater than y, whilst in those rats which develop eye disease before death x is less than y. Such a difference in the requirement of young animals of the same species is surprising. The facts may mean no more than that such a variation in individual resistance exists as is seen clinically when one element in a symptom complex is more prominent in one case, and some other element in another. But under the controlled conditions of experimental studies such variations within the same species are not usually seen.

An alternative hypothesis which suggests itself is that two factors are involved; one responsible for life and growth and another necessary for the nutritive integrity of a special tissue such as the cornea. Rats, which at the start of the experiment contain a relatively larger supply of the life-growth factor develop eye disease before death, whilst those which start with relatively more of the corneal-protecting factor exhaust their life-growth factor (*i.e.* die) before developing eye disease. Such a hypothesis presupposes that these two factors are present in varying proportions in different natural foodstuffs, and that the relative amounts of each accumulated in the rat at the beginning of the experiment are conditioned by its previous range of diet. The final consideration of these points must be postponed until they can be considered in relation to the histological and bacteriological results.

B. HISTOLOGICAL AND BACTERIOLOGICAL.

We have been unable to find in the literature any detailed description of keratomalacia among rats, and have therefore attempted an investigation of this disease. In planning this work the questions we had in view were as follows:

1. What microscopic degenerative changes take place in the eye during the course of this disease?

2. Are these changes directly and solely caused by bacterial invasion, or is this infection preceded by histological changes in the tissue brought about by a prolonged course of a diet deficient in the fat-soluble factor?

3. Is one particular organism predominant in the bacterial infection?

B (1). Histological.

Preparations have been made of the eyes of:

A. Normal rats on mixed diet.

B. Rats which though on a diet deficient in the fat-soluble factor showed none of the symptoms of eye disease.

C. Rats in which symptoms of disease were apparent in one eye but not in both.

D. Rats in which both eyes were in an advanced stage of the disease.

E. Rats in which a cure had been effected and sight restored by change of diet, *i.e.* by the addition of the fat-soluble factor.

F. Rats in which change of diet had cleared up the purulent symptoms and effected the healing and regeneration of tissue, but in which sight had not been restored.

Method.

The eyes were placed in 10 % formalin for 24 hours, washed in water, passed through the alcohols and xylene, and embedded in paraffin. Sections were stained by each of the following:

1. Ehrlich's haematoxylin and eosin.
2. Iron haematoxylin and van Gieson.
3. Gram-Weigert stain for bacteria.

Results.

Figs. 9–18 will be found on Plates IX and X.

A. *Normal rats on mixed diet.*

Case 1. Fig. 17 shows a section of a normal eye; Fig. 9 shows a section of a normal cornea.

B. *Rats which though on a diet deficient in the fat-soluble factor showed none of the external symptoms of eye disease.*

Case 2. *Rat No.* 53 ♀.

Diet. Laboratory synthetic diet with palm kernel oil extract of yeast and lemon juice.

Growth curve, see Fig. 3.

Note that the growth and life of this rat were unusually prolonged and that no symptoms of eye disease were shown at any time. The rat was killed on the 88th day. Sections of the cornea of this rat do not differ in any important respect from those of the normal cornea so that it is unnecessary to append a figure.

C. *Rats in which symptoms were apparent in one eye but not in both.*

Case 3. *Rat No.* 7.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

This case is one in which symptoms were apparent in one eye whilst the other seemed to be normal.

Histological examination of the apparently normal eye (Fig. 10) shows the presence of a few leucocytes (*l*) in the cornea proper.

Sections of the other eye show a swollen cornea with loosened tissue due to the infiltration of fluid; leucocytes are present in the cornea proper.

Case 4. *Rat No.* 74.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

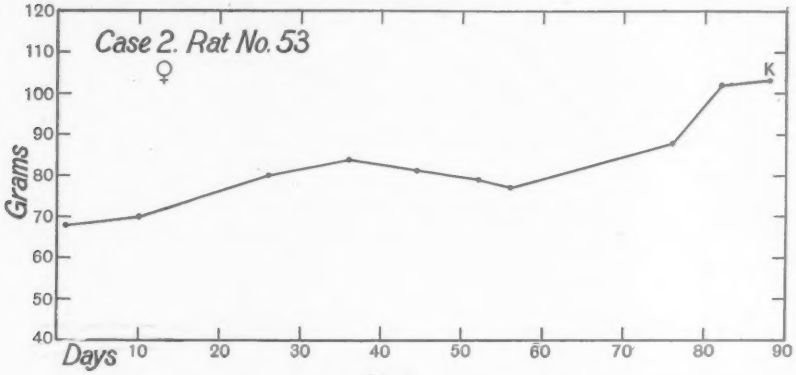


Fig. 3.

As in Case 3 (Rat No. 7) one eye was visibly diseased whilst the other was apparently normal. Histological examination of the normal eye (Fig. 11) shows that, unlike the apparently normal eye in Case 3, no abnormality can be discerned in the cornea.

In the case of the diseased eye of the same rat (Fig. 12) the cornea shows an enormously increased number of corneal corpuscles and a great many well-defined blood-vessels lined with epithelium (*b.v.*). These profound tissue changes are not uniformly distributed throughout the cornea but are more marked in some areas than in others. This observation will be referred to later in the discussion of the significance of the histological changes.

D. *Rats in which both eyes are in an advanced stage of the disease.*

Case 5. *Rat No.* 77.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, see Fig. 4; symptoms of disease were first noted on the 66th day (A).

Both eyes in this rat were in approximately the same state and show apparently an earlier stage of the disease than does the diseased eye of Case 4.

In Fig. 13 we notice localised increase of corneal corpuscles with well-marked blood vessels (*b.v.*). As in Case 4 these changes are not uniformly distributed throughout the cornea.

Gram-stained preparations of both these eyes show large numbers of Gram-positive cocci of the pneumococcal type, see Fig. 14 (*d*).

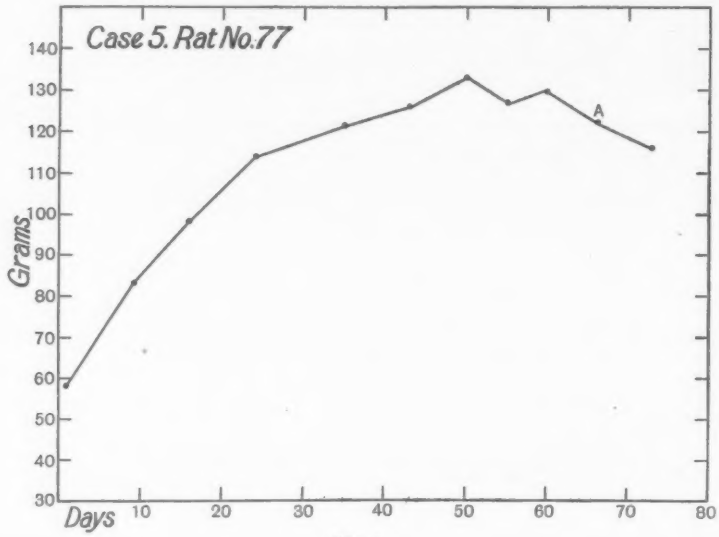


Fig. 4.

Case 6. *Rat No.* 60.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

The first symptoms of disease were noted on the 51st day (A); death took place on the 82nd day.

Growth curve, see Fig. 5.

Both eyes of this rat were in an advanced stage of the disease; both corneas were completely disintegrated exposing the front of the lens, which protruded during life.

Sections of the eye showed the lens complete but no anterior structure remaining.

Gram-stained preparations showed the presence of Gram-positive cocci of the pneumococcal type in the sclerotic.

E. *Rats in which a cure has been effected and sight restored by the addition to the diet of a fat containing the fat-soluble vitamine in place of the palm kernel oil.*

Case 7. *Rat No.* 113 ♀.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, see Fig. 6.

In order to interpret this growth curve it is necessary to give details of the history of this rat.

The first symptoms of eye disease were noted on the 50th day of experiment, the left eye only being affected (A).

On the 54th day both eyes were affected; the palm kernel oil was then replaced by bone marrow in order to test the vitamine content of this fat (B).

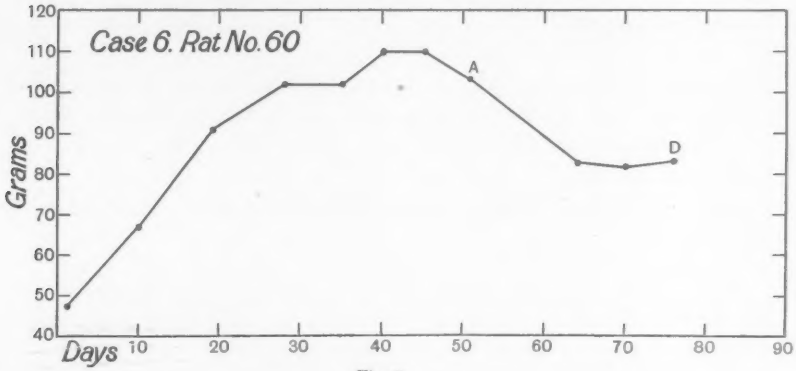


Fig. 5.

On the 57th day the eyes had improved (C), and on the 66th day all purulent symptoms had disappeared and the eyes were apparently normal (D).

Symptoms of eye disease returned however on the 93rd day both eyes becoming purulent (E).

On the 96th day the symptoms were more pronounced (both eyes were then swabbed) (F).

On the 105th day the bone marrow was replaced by butter (G).

On the 119th day the eyes were cured (H).

On the 137th day the rat was killed (K).

It is seen from the above notes that 18 days elapsed between the apparent completion of the cure by butter and the killing of the rat.

Histological examination of the sections showed that the cornea had completely recovered the normal state.

Case 8. *Rat No.* 114 ♀.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, Fig. 7.

The first symptoms of eye disease were noted on the 72nd day when both eyes were slightly purulent (Z). On the 82nd day both eyes were very purulent and closed (Y). On the 85th day crude light petroleum extract of carrot was added to the palm kernel oil (X); on the 95th day a marked improvement had taken place, a slight discharge only from the right eye being noticeable (W).

On the 98th day the eye was swabbed.

On the 107th day both eyes were clean showing only minute spots of discharge on the lid (V); these had disappeared on the 119th day (U). On the 142nd day the rat was killed (K).

As in Case 7, a considerable interval, in this case 23 days, elapsed between the completion of the cure and the killing of the rat. Histological examination of the sections showed that the cornea had almost completely resumed its normal condition, a slight increase in the number of corneal corpuscles only being still apparent.

F. *Rats in which supply of the fat-soluble factor had cleared up the purulent symptoms and effected the healing and regeneration of the tissue, but in which sight had not been restored.*

Case 9. *Rat No.* 5.

Diet. Laboratory synthetic diet with palm kernel oil, extract of yeast and lemon juice.

Growth curve, see Fig. 8.

The earliest symptoms of eye disease in this rat escaped observation. On the 39th day both eyes were opaque and one was bleeding (A). Light petroleum extract of carrot was then added to the palm kernel oil. On the 46th day (B), purulent symptoms had disappeared; the left eye was clean and whole but with an opaque cornea; the right eye was permanently closed. On the 251st day the rat was killed.

Sections of the left eye disclose striking abnormalities. The cornea (Fig. 15) is considerably flattened and large vessels full of blood cells (*r.b.c.*) still remain. Since 90 days elapsed between the completion of the cure and the killing of the rat, this condition of the cornea may be regarded as permanent and not as representing a stage in the cure. In the case of the right eye the cornea degenerated so completely that the lens actually fell out of the eye during life; when the diet was changed the purulent condition of the eye was cured. Histological examination of the sections (Fig. 18) shows that the disappearance of the large lens has caused collapse and shrinkage of the "eye"; in place of the cornea there is a curious regenerated tissue built up from various elements (Fig. 16). This opaque anterior structure contains an enormous number of corneal corpuscles and a mass of fibrous connective tissue that has been built up from behind by the proliferation of corneal corpuscles.

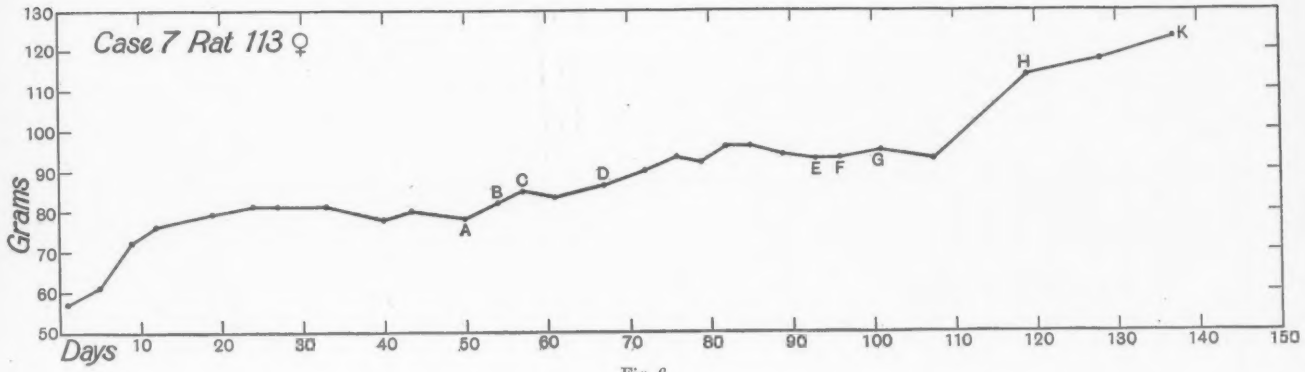

Case 7 Rat 113 ♀
Grams
Days
A
B
C
D
E
F
G
H
K

Fig. 6.

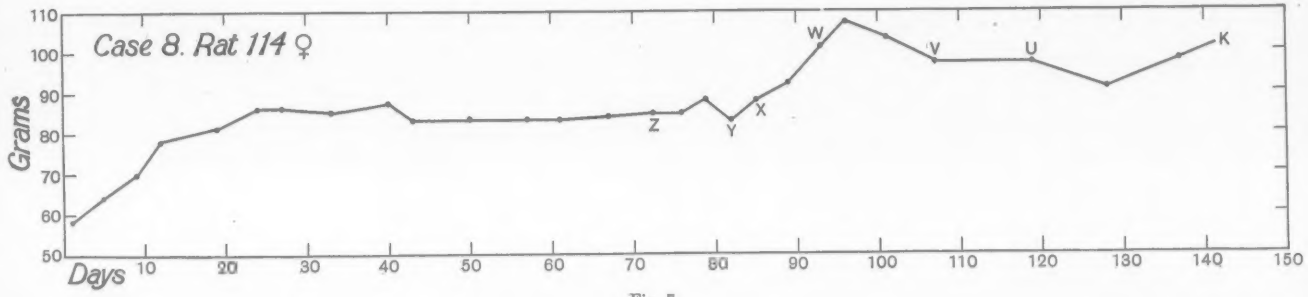

Case 8. Rat 114 ♀
Grams
Days
W
V
U
K
Z
Y
X

Fig. 7.

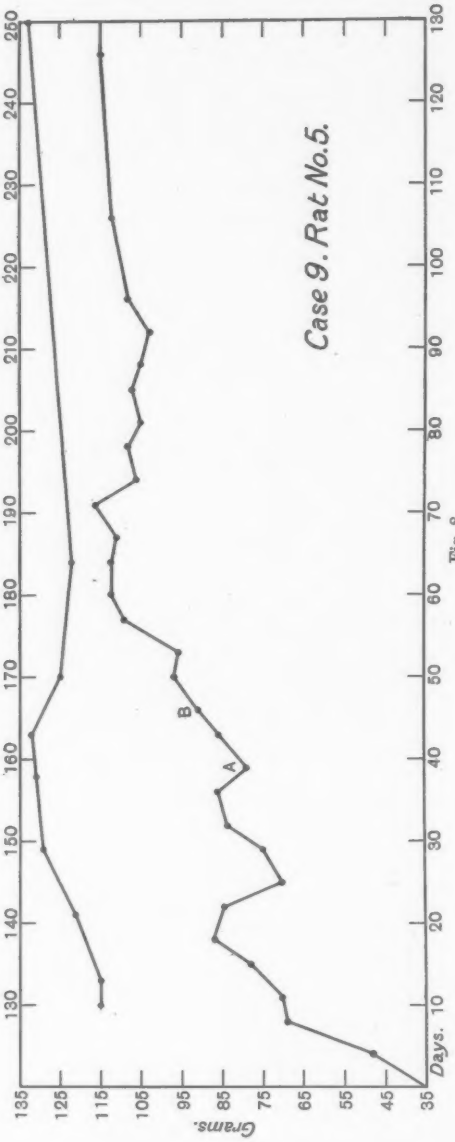

Case 9. Rat No. 5.
Grams.
Days.
A
B
135
125
115
105
95
85
75
65
55
45
35
10
20
30
40
50
60
70
80
90
100
110
120
130
130
140
150
160
170
180
190
200
210
220
230
240
250

Fig. 8.

Summary of Histological Results.

The earliest histological changes noticed in the cornea are those in Case 3 (the apparently normal eye of a rat whose other eye was purulent and degenerated). This apparently normal eye showed the presence of leucocytes in the cornea, a condition which is frequently associated with incipient bacterial infection. We cannot therefore deduce from the condition of such corneas that there is any demonstrable histological change directly attributable to dietary deficiency prior to bacterial invasion. A slightly later stage is shown by the other eye described in Case 3, where purulent symptoms were apparent and the cornea was swollen and oedematous. Again leucocytes are present, but no definite blood vessels are yet discernible.

In Case 5 very definite blood vessels are present, and there is marked increase in the number of corneal corpuscles.

The bad eye of Case 4 shows the same abnormalities in a much greater degree. In both Cases 4 and 5 the tissue changes are more or less localised in definite areas, thus affording strong evidence that they are the direct result of bacterial infection.

In Case 6 the cornea is completely degenerated and here, as in Case 5, organisms of the pneumococcal type were demonstrated.

The histological conditions of "cured" eyes are of different types. In the majority of cases where cure is effected by change of diet, sight is restored and the cornea completely regains its normal condition as in Cases 7 and 8.

Where degeneration has (presumably) proceeded further before cure is commenced, the cornea may remain opaque as in Case 9, left eye. Histological investigation of this cornea shows that it is flattened and contains many large blood vessels. As 105 days had elapsed between the apparent completion of the cure and the killing of the rat, it is justifiable to assume that this condition was permanent and did not represent a stage in the recovery of the cornea.

In some cases the cornea has so far degenerated before cure is begun that the lens is forced through the aperture during life, and "cure" consists in the disappearance of pus and the healing over of the injured tissues. Case 9, right eye, exemplifies this condition.

B (2). Bacteriological.

Method.

Pus was collected from rats' eyes with a sterile platinum loop, direct films were examined, and cultures were put up in tryptic broth ($P_H = 7{\cdot}4$), on tryptic blood agar ($P_H = 7{\cdot}4$), and on inspissated serum. Anaerobic broth cultures were also incubated, but in no case did they yield the growth of organisms other than those facultative anaerobes that also appeared in the aerobic cultures. The organisms were obtained in pure culture, and fermentation reactions were studied by growth for seven days in litmus milk and

sugars, etc. (1 % of the substance dissolved in diluted tryptic broth, with litmus as an indicator). The organisms present in the conjunctival sac of several normal and "cured" rats were examined in a similar way.

Results.

The following organisms were isolated:

1. Pneumococci; two types (see Table IV).
2. Staphylococci (*S. albus* and *S. aureus*).
3. Gram-positive cocco-bacilli; four types (see Table IV).
4. Bacillus subtilis.
5. Gram-positive diphtheroids; two types (see Table IV).
6. Other Gram-positive bacilli; two types (see Table IV).
7. Gram-negative bacilli; two types (see Table IV).

A list of the organisms found in 18 cases is appended:

α. Normal rats on mixed diet; eyes clean and healthy.

Case 10. (*a*) S. aureus.
(*b*) Cocco-bacillus, Type III.

Case 11. (*a*) Pneumococcus, Type I.
(*b*) Gram-positive bacillus, Type II.

Case 12. (*a*) S. albus.

β. Rats on diet deficient in fat-soluble factor.

Case 13. *Rat No.* 91; eyes purulent and bleeding.
(*a*) Pneumococcus, Type I.
(*b*) Cocco-bacillus, Type II.

Case 14. *Rat No.* 129; eyes closed; purulent.
(*a*) Pneumococcus, Type I.
(*b*) S. aureus.

Case 15. *Rat No.* 48; eyes purulent.
(*a*) Pneumococcus, Type I.
(*b*) Diphtheroid, Type I.

Case 16. *Rat No.* 202; eyes purulent.
(*a*) Pneumococcus, Type II.
(*b*) Cocco-bacillus, Type III.

Case 17. *Rat No.* 205; eyes bleeding.
(*a*) Pneumococcus, Type II.
(*b*) Gram-negative bacillus, Type II.

Table IV.

Organism	Glucose	Saccharose	Mannitol	Maltose	Lactose	Raffinose	Inulin	Levulose	Galactose	Dextrin	Dulcitol	Milk	Notes
Pneumococcus, Type I	A	A	A	A	A	A	A	A	A	A	—	A	Long chains formed in broth.
„ „ II	A	A	A	A	A	—	A	A	A	A	—	A or AC	Long chains formed in broth.
Cocco-bacillus, Type I	A	A	—	A	A	A	—	A	A	A	—	A	Very small colonies on agar.
„ „ II	A	A	A	A	A	—	A	A	A	A	—	AC	Strong growth on agar.
„ „ III	A	(A)	A	A	A	—	—	A	A	A	—	(AC)	Spreading greenish growth on agar.
„ „ IV	A	—	—	—	—	—	—	(A)	A	(A)	—	AC	Strong white viscous growth on agar; pellicle formed on broth.
Diphtheroid, Type I	No growth												Very small colonies on agar.
„ „ II	A	—	—	—	—	—	—	A	A	A	—	—	Strong growth on agar.
Gram-positive Bacillus, Type I	A	A	—	A	—	—	—	A	—	A	—	—	White lustrous growth on agar.
Gram-positive Bacillus, Type II	A	A	A	A	A	A	—	A	A	A	—	A	Strong growth on agar; non-motile.
Gram-negative Bacillus, Type I	—	—	—	—	—	—	—	A	A	A	—	A	Liquefies inspissated serum.
Gram-negative Bacillus, Type II	AG	—	—	—	—	—	—	AG	AG	AG	—	A	Strong growth on agar; non-motile.

A = acid.
AG = acid and gas.
AC = acid and clot.
(A) = acid usually but not invariably formed.

Case 18. *Rat No.* 63; eyes purulent.
(*a*) Gram-positive lancet-shaped diplococci not obtained in pure culture.
(*b*) S. albus.
(*c*) B. subtilis.

Case 19. *Rat No.* 113 (see Case 7); eyes purulent.
(*a*) S. aureus.
(*b*) B. subtilis.
(*c*) Gram-negative bacillus, Type I.

Case 20. *Rat No.* 201; eyes purulent.
(*a*) Diphtheroid, Type I.

Case 21. *Rat No.* 203; eyes opaque and bleeding; no pus.
(*a*) Cocco-bacillus, Type III.
(*b*) Gram-positive bacillus, Type I.

Case 22. *Rat No.* 204; eyes purulent and bleeding.
(*a*) Cocco-bacillus, Type III.
(*b*) Diphtheroid, Type I.

Case 23. *Rat No.* 206; eyes closed; slightly purulent.
(*a*) Cocco-bacillus, Type IV.
(*b*) Diphtheroid, Type I.

Case 24. *Rat No.* 207; eyes purulent.
(*a*) Cocco-bacillus, Type III.
(*b*) Cocco-bacillus, Type IV.

γ. Rats which developed eye disease on a diet deficient in the fat-soluble factor, and which were subsequently cured by change of diet.

Case 25. *Rat No.* 114 (see Case 8).

Right eye still purulent.
(*a*) Pneumococcus, Type I.
(*b*) S. aureus.
(*c*) B. subtilis.
(*d*) Gram-negative bacillus, Type II.

Left eye: no pus.
(*a*) Diphtheroid, Type II.

Case 26. *Rat No.* 161; eyes still showing symptoms of disease.
(*a*) S. albus.
(*b*) Diphtheroid, Type II.

Case 27. *Rat No.* 63 (see Case 1 before treatment).

The diet was changed on the 73rd day, and on the 78th day the eyes were quite clean. Two days later the eyes were swabbed.
(*a*) Cocco-bacillus, Type I.

An attempt was made to determine whether the eyes of rats which had subsisted for a prolonged period on a diet deficient in the fat-soluble factor were any more susceptible to bacterial infection than the eyes of control rats on butter diet. For this purpose four rats were selected, two normal rats and two which had not developed eye disease although they had been eight weeks on a diet deficient in the fat-soluble factor. The right eye of each of these four rats was smeared with pus just removed from a purulent eye. The experiment was repeated four times and in each case no symptoms of eye disease developed in any of the four rats.

Interpretation of Bacteriological Results.

Cases 10, 11 and 12 indicate that the normal conjunctiva of the rat has a varying flora. When interpreting the results from diseased eyes, it must be remembered that pus from the eye is open to contamination from the air, and that therefore an organism isolated from the pus is not *necessarily* concerned with the production of lesions in the eye.

Pus from the diseased eyes in many cases (13, 14, 15, 16, 17, 25) showed the presence of a Pneumococcus, but this organism was not invariably present, in fact no organism that we obtained was common to all the purulent cases, so that we have not been able to show that the specificity of the disease is related to bacterial species.

From the results given above, it appears that when the rat has been on a deficiency diet for such a length of time that the resistance of the cornea is affected, those pathogenic bacteria which happen to be present in the conjunctival sac invade the corneal epithelium and induce the destruction of tissue.

Summary and Discussion.

The histological and bacteriological evidence shows that keratomalacia among rats consists in a breakdown of the corneal tissue, caused by bacterial invasion.

The dietetic and statistical evidence shows that bacterial invasion of the cornea occurs only after a prolonged course of diet in which the fat-soluble factor is absent. The condition therefore directly attributable to dietetic deficiency is a predisposition to bacterial infection of the cornea leading to purulent symptoms and the destruction of tissue.

The question then to be decided is whether this predisposition of the cornea to infection is to be regarded as a true deficiency disease. The answer to this question is complicated by the following consideration.

We have hitherto failed to demonstrate with certainty any histological change in the cornea preceding bacterial invasion. We cannot therefore state the precise moment at which the predisposition to infection begins. The only criterion we possess for determining the preliminary change caused by the deficient diet is the appearance of the secondary symptoms caused by bacterial

invasion. Supposing a variable time to elapse between the preliminary change in the cornea and the actual invasion by bacteria—inflammation, pus, etc.—then the statistics collected in the earlier part of the paper are invalidated, although the evidence on the question of the cure is unaffected. If however we may assume that the organisms normally present in the conjunctival sac ensure the infection of the cornea as soon as the absence of the fat-soluble factor has rendered it liable to infection, then we are justified in regarding, for statistical purposes, the appearance of the purulent symptoms as an immediate notification of the preliminary change in the cornea due to dietary deficiency, and the evidence adduced on the incidence of the disease remains valid.

Assuming—as seems probable—the latter hypothesis to be the true one, the statistical evidence described in the earlier part of our paper can be applied to decide whether predisposition of the cornea to infection is a true deficiency disease. The conclusions then arrived at may be briefly recapitulated.

1. Corneal disease is not coincident with failure of growth culminating in death; it forms only 28 % of the cases examined.

2. This disease is further differentiated from the cessation of growth-death symptoms by occurring at a later stage in the experimental period and rising more rapidly to a maximum.

3. At no period in the experiment are all the deaths preceded by eye disease or all the survivors afflicted with it.

4. Cure of the corneal disease was effected by the replacement of the fat-soluble factor in 100 % of the cases attempted.

Before this evidence, however, serves to place preliminary deterioration of the cornea among deficiency diseases, it requires the acceptance of one of the three supplementary hypotheses A, B or C below:

either

A. The symptoms caused by experimental fat-soluble deficiency disease among rats vary in such a way that in some cases the cessation of growth-death symptoms predominate to such a degree that death ensues before the deterioration of the cornea commences, whereas in other cases the nutritive integrity of the cornea is disturbed before the cessation of growth symptoms reach an acute stage;

or

B. The concentration of the fat-soluble factor in the tissues of the rat necessary to protect the cornea was less than that required for life and growth in 72 % of the cases examined (*i.e.* in those dying without eye disease); whilst the reverse was true in 28 % of the cases (*i.e.* in those developing disease before death);

or

C. Two factors are involved; one responsible for the continuance of growth and the maintenance of life, and another for the protection of the cornea.

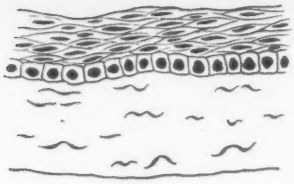


Fig. 9

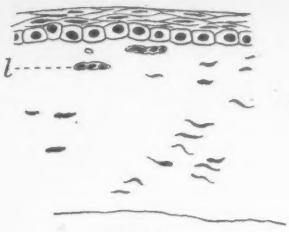
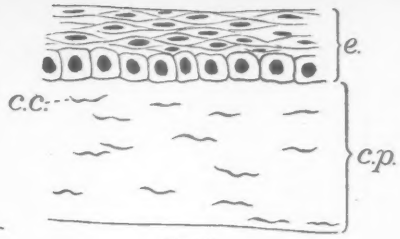


Fig. 10



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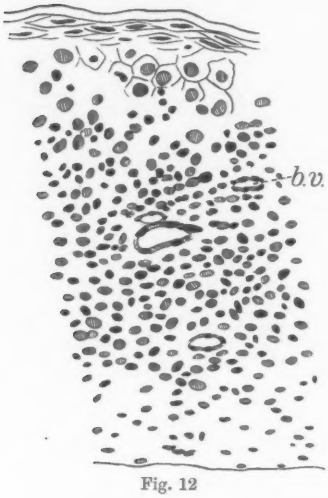
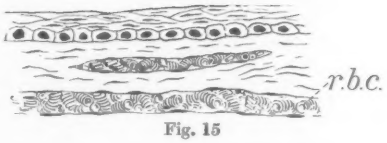


Fig. 12



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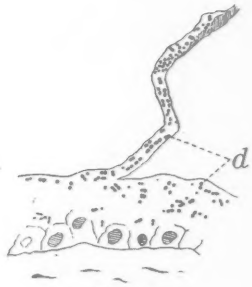


Fig. 14

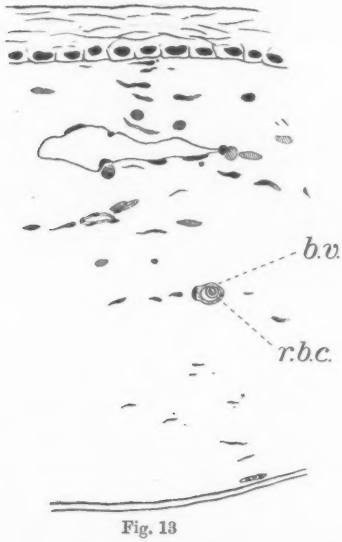


Fig. 13

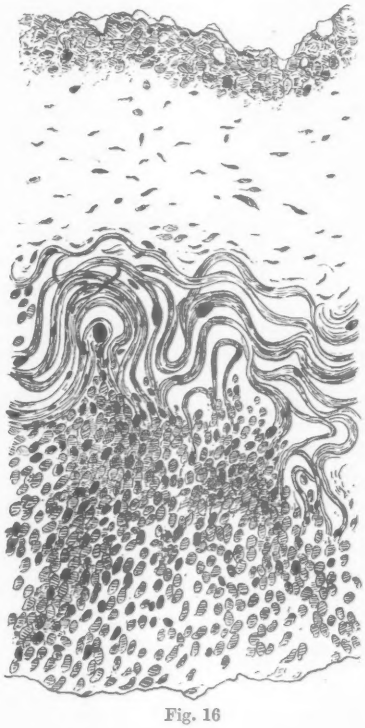
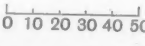


Fig. 16



Figs. 9, 10, 11, 12, 13, 15, 16.

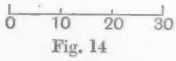


Fig. 14

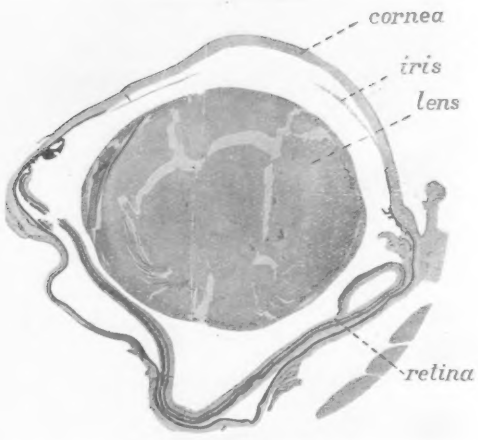


Fig. 17

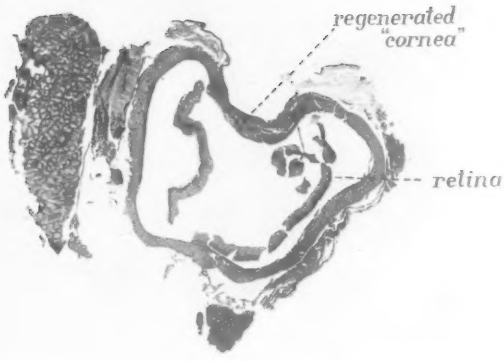


Fig. 18

The authors gladly avail themselves of this opportunity for thanking Professor Hopkins for his stimulating help and advice in the course of this investigation. They are also deeply indebted to Dr H. K. Anderson, Master of Gonville and Caius College, Cambridge, for his invaluable help in interpreting histological evidence.

The expenses of this research were defrayed from funds contributed by the Planters Margarine Co., and the Maypole Co.

The authors are also indebted to the above firms for their courtesy in permitting the results to be published.

EXPLANATION OF PLATES IX AND X.

Fig. 9. Normal cornea.
Fig. 10. Case 3, Rat 7. Cornea of apparently normal eye.
Fig. 11. Case 4, Rat 74. Cornea of normal eye.
Fig. 12. Case 4, Rat 74. Cornea of diseased eye.
Fig. 13. Case 5, Rat 77. Cornea.
Fig. 14. Case 5, Rat 77. Portion of degenerated corneal epithelium stained by Gram's method.
Fig. 15. Case 9, Rat 5. Cornea of left eye.
Fig. 16. Case 9, Rat 5. Cornea of right "eye."
Fig. 17. Microphotograph of normal eye. ($\times 25$.)
Fig. 18. Microphotograph of Case 9, Rat 5, right "eye." ($\times 25$.)

b.v. = blood vessel. *c.c.* = corneal corpuscle. *c.p.* = cornea proper. *d.* = Gram-positive diplococci. *e.* = epithelium. *l.* = leucocytes. *r.b.c.* = red blood corpuscles.

Microphotographs by A. C. P. Lunn, King's College, Cambridge.

REFERENCES.

Bloch, C. E. (1917). *Ugeskrift for Laeger*, **79**, 349.
Bulley, E. C. (1919). *J. Biol. Chem.*, **13**, 103.
McCollum and Simmonds (1917, 1). *J. Biol. Chem.*, **32**, 181.
—— (1917, 2). *J. Biol. Chem.*, **32**, 347.
Mori, M. (1904). *Jahrb. Kinderheilk*, **59**, 175.

XLIII. BARGER'S MICROSCOPICAL METHOD OF DETERMINING MOLECULAR WEIGHTS.

PART II. ITS APPLICATION TO CASEINOGEN.

By KUMAO YAMAKAMI.

From the Lister Institute of Preventive Medicine, London.

(*Received April 12th, 1920.*)

A. The molecular and ionic concentration of alkali caseinogenate solutions of neutral and alkaline reaction.

This work has been undertaken to study the osmotic concentration of alkali caseinogenate solutions. T. B. Robertson [1909, 1918, p. 336] and T. B. Robertson and T. C. Burnett [1909] have investigated the cryoscopic behaviour of dissolved caseinogenates of alkalies and alkaline earths (NH_4OH, KOH, LiOH, $Ca(OH)_2$ etc.) and found that:

"Keeping the concentration of casein constant and increasing the concentration of base bound by the caseinogen results in a proportionate increase in the observed depression, in other words, the molecular and ionic concentration of caseinate solutions is conditioned by the combined alkali. On the other hand, the increasing of the quantity of dissolved casein does not alter the observed depression in any appreciable degree when casein is dissolved in the solution of base in the proportion of 80.10^{-5} gram equivalent or 50.10^{-5} gram equivalent base per gram casein."

From these experimental results they concluded that each equivalent of combined base yields the same number of protein ions derived from the splitting of successive .CO.NH groups, and suggested that "if this relation were maintained for other concentrations, then at zero concentration of combined base, if casein were soluble under such conditions, the freezing point depression due to dissolved casein would be zero. The possibility is therefore indicated that base- and acid-free protein may exert an immeasurably small osmotic pressure, probably owing to the polymerisation of protein as the uncombined protein is set free."

When I was in the laboratory of Toronto University, it was suggested that I should apply Barger's microscopical method of determining molecular weights to caseinogen. Accordingly I investigated the osmotic concentrations of alkali caseinogenate solutions employing caseinogen which was prepared by a colleague, but I could not demonstrate the parallelism between the

molecular ionic concentrations of caseinogenate solution and the concentration of the base in which the caseinogen was dissolved.

Prof. Robertson suggested that this might be due to the impurity of the caseinogen employed, and this proved to be the case. For, when I repeated the experiments at the Lister Institute, London, with "Hammarsten casein" (Kahlbaum) consistent results were obtained.

The molecular and ionic concentrations of Na-caseinogenate solutions have been observed to be absolutely dependent upon the concentration of NaOH, not only when the proportion of base to caseinogen is between $80 . 10^{-5}$ and $50 . 10^{-5}$ g. equivalents base per g. caseinogen, but also for all other ratios between $180 . 10^{-5}$ and $50 . 10^{-5}$ g. equivalents.

Experiment 1. "Hammarsten casein" (Kahlbaum) was purified according to the directions of Robertson [1918, p. 39] and was dissolved in NaOH solutions of varying concentrations, changing the amount of caseinogen, so that the ratio of caseinogen to base varied between $180 . 10^{-5}$ and $50 . 10^{-5}$ g. equivalents base per g. caseinogen and the molecular and ionic concentrations of these caseinogenate solutions were determined by means of Barger's method, employing urea as the standard substance.

Unfortunately there is at present no good test for the purity of caseinogen, but its degree of purity in my experiments is indicated by the following results of the tests which are generally applied.

1. A perfectly white powder which is absolutely dry.
2. Its solution is colourless.
3. The percentage of ash is 0·11.
4. The solution in alkali in the proportion of $80 . 10^{-5}$ g. equivalent is just neutral to phenolphthalein.
5. The solubility in 5 % NaCl solution is less than 2 %.

If van Slyke and Bosworth's statement [1913] that the usual caseinogen preparations obtained from chemical supply houses are generally contaminated with water-insoluble calcium caseinogenate (monobasic caseinogenate) and calcium phosphate is true, the directions for purification given by Robertson cannot be very effective, but the solubility in NaCl solution as well as the content of ash in the caseinogen preparation employed in my experiments indicate that it is sufficiently pure for the purpose.

The purity of the urea employed as standard had been checked by determining the molecular weights of other pure substances such as cane-sugar, lactose, etc. with its aid.

The first measurement of the drops was made 30–40 minutes after the preparation of the tubes and the second determination after standing for 24 hours in a cold room.

The caseinogenate solutions in these experiments had generally small molecular-ionic concentrations, consequently the change of the drops was very slight even when the tubes were exposed to ordinary room temperature. Nevertheless the tubes were kept cool in the vestibule of a cold room where

the temperature was about 10°–11° C., because it was feared that the decomposition of caseinogen by micro-organisms which cannot be prevented from entering the solution, might take place more easily at room temperature.

The technique employed is exactly the same as described in Part I [Yamakami, 1920], the directions of Barger being strictly followed. In the first determination the absolute measurements of the drops and the changes in them are given by way of illustration. In all the remaining determinations only the concentrations of the solutions in the limiting tubes are given.

In the first column of the table, the molar concentration of the urea solutions is given, and the drops denoted by odd numbers are those of the caseinogenate solutions.

Table I.

(*a*) NaOH = *N*/20, caseinogen = 10 %; alkali: caseinogen = 50 . 10^{-5} g. eq.: 1 g.

Urea	I	II	III	IV	V
M/12	221 – 7	384 + 7	235 – 7	359 + 11	303 – 10
M/14	251 – 6	279 + 6	270 – 15	331 + 7	209 – 9
M/16	402 – 1	676 + 2	175 – 4	754 + 4	608 – 4
M/20	388 + 2	555 – 1	490 + 1	612 ± 0	224 ± 0
M/25	504 + 12	841 – 1	785 + 5	670 + 4	492 + 2

Limits = 1/16 – 1/20 *M*.

(*b*) NaOH = *N*/20, caseinogen = 8 %; alkali: caseinogen = 62 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*c*) NaOH = *N*/20, caseinogen = 6·2 %; alkali: caseinogen = 80 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*d*) NaOH = *N*/20, caseinogen = 4 %; alkali: caseinogen = 125 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*e*) NaOH = *N*/20, caseinogen = 2·78 %; alkali: caseinogen = 180 . 10^{-5} g. eq.: 1 g.
Limits = 1/16–1/20 *M*.

(*f*) NaOH = *N*/20·8, caseinogen = 6 %; alkali: caseinogen = 80 . 10^{-5} g. eq.: 1 g.
Limits = 1/17·5–1/20 *M*.

(*g*) Caseinogen solutions containing respectively 50 . 10^{-5} and 80 . 10^{-5} g. equivalent alkali per g. caseinogen were compared directly.

I	II	III	IV	V
–2	–2	–3	–3	–2
–2	+1	+1	+1	+1
–3	–2	–2	–2	–1

The drops denoted by even numbers are those of caseinogen solution containing 50 . 10^{-5} g. equivalent alkali.

Table II.

(*a*) NaOH = *N*/25, caseinogen = 8 %; alkali: caseinogen = 50 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*b*) NaOH = *N*/25, caseinogen = 6 %; alkali: caseinogen = 66 . 7·10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*c*) NaOH = *N*/25, caseinogen = 5 %; alkali: caseinogen = 80 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*d*) NaOH = *N*/25, caseinogen = 3·75 %; alkali: caseinogen = 106·6 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

(*e*) NaOH = *N*/25, caseinogen = 2·22 %; alkali: caseinogen = 180 . 10^{-5} g. eq.: 1 g.
Limits = 1/20–1/25 *M*.

Table III.

(*a*) NaOH $\doteq N/33$, caseinogen = 6 %; alkali: caseinogen = $50 . 10^{-5}$ g. eq.: 1 g.
Limit = 1/35 *M*.

(*b*) NaOH = $N/33$, caseinogen = 3·75 %; alkali: caseinogen = $57{\cdot}6 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/35–1/40 *M*.

(*c*) NaOH = $N/33$, caseinogen = 1·7 %; alkali: caseinogen = $182 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/30–1/35 *M*.

(*d*) NaOH = $N/50$, caseinogen = 4 %; alkali: caseinogen = $50 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/40–1/50 *M*.

(*e*) NaOH = $N/50$, caseinogen = 3·7 %; alkali: caseinogen = $53 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/40–1/50 *M*.

(*f*) NaOH = $N/50$, caseinogen = 2·5 %; alkali: caseinogen = $80 . 10^{-5}$ g. eq.: 1 g.
Limit = 1/40 *M*.

(*g*) NaOH = $N/50$, caseinogen = 2·2 %; alkali: caseinogen = $90 . 10^{-5}$ g. eq.: 1 g.
Limits = 1/40–1/50 *M*.

The results obtained in the above tables are so regular that detailed explanation and discussion are hardly necessary. The molecular and ionic concentrations of the caseinogenate solutions were always very nearly identical with the concentrations of the alkali solutions in which the caseinogen was dissolved, as long as the proportion of alkali to caseinogen was between $180 . 10^{-5}$ g. equivalent and $50 . 10^{-5}$ g. equivalent base per g. caseinogen.

The only thing I feared was that urea might combine chemically with the alkali of the caseinogenate solutions. But this, very probably, would not occur because the urea solution is very slightly acid to phenolphthalein, while the acidity of caseinogen is pretty strong. Thus the urea solution would not be able to attract the alkali which was combined with such a strong acid as caseinogen. This would be particularly true when the reaction of the caseinogenate solution itself was strongly acid to phenolphthalein. Moreover the regularity of the change of the drops suggests that no chemical reaction was taking place between the two solutions.

As to the effect of the impurities in the caseinogen upon the results obtained, the calcium caseinogenate which is alleged to contaminate most preparations of caseinogen would not exert much influence upon the molecular and ionic concentrations.

The possible impurities which must be taken into consideration in these experiments are crystalloid substances which have small molecular weights, because they would change the osmotic concentrations of the caseinogenate solutions to a considerable extent even if present only in small quantities. In order to get rid of this objection, I dialysed my caseinogenate solutions and convinced myself that the caseinogen used was free from crystalloid substances since no lowering of the molecular and ionic concentration of the caseinogenate solution resulted from dialysis. Reference will be made to these experiments later.

On one occasion, the direct comparison of caseinogenate solutions which contained varying amounts of caseinogen and alkali gave the same result,

and this indeed might well be expected, as in the last experiment recorded in Table I, in cases where the reaction of the two caseinogenate solutions compared did not differ very much. But when there existed large differences in the reactions of the two solutions, the change of the drops was quite irregular, so that no conclusion could be drawn from the experiment. This is probably due to the chemical attraction between the two solutions, which interferes with the regularity of movement of the solution from one drop to another because of the physical attraction of solute for solvent.

It is my belief that the observation made by Robertson and Burnett by means of the cryoscopic method is satisfactorily confirmed by my experiments in which Barger's method was employed.

Now, if this relationship between alkali, caseinogen and the molecular ionic concentration of the solution is maintained unchanged up to the highest percentage of caseinogen which is soluble in a given amount of alkali, then the largest molecular ionic weight, we can expect to obtain by Barger's method, must be dependent upon the solubility of caseinogen in the alkaline solution.

If caseinogen is soluble in the alkaline solution in the proportion of $25 . 10^{-5}$ g. equivalent of alkali per g. caseinogen then we should obtain 4000 as the molecular or ionic weight of caseinogen, if, on the other hand, the solubility of caseinogen is $12{\cdot}5 . 10^{-5}$ g. equivalent then the figure would become 8000 provided that the osmotic concentration of caseinogenate solution is always conditioned by the concentration of alkaline solution.

The solubility was, therefore, studied before I undertook the investigation of the molecular and ionic concentrations of the alkali caseinogenate solutions of acid reaction in which the proportion of alkali to caseinogen was smaller than $50 . 10^{-5}$ g. equivalent per g. caseinogen.

B. The solubility of caseinogen in alkali solution and the molecular weight of caseinogen calculated from the solubility or the combining capacity.

The solubility of caseinogen in alkali solution or "the combining capacity of casein at saturation of the base with casein, that is when the base has dissolved the maximum amount of casein which it will dissolve," as expressed by Robertson, has recently been stated to be $11{\cdot}4 . 10^{-5}$ g. equivalent alkali per g. caseinogen. This is based upon two experimental results, the one, obtained by Robertson, and the other, by van Slyke and Bosworth [1913], and it is supported by deduction from the formula for the lowering of the conductivity of alkali solution which is caused by dissolved caseinogen.

Robertson found that the relation between b_1, the concentration of alkali solution in which caseinogen is dissolved, and Δ, the lowering of its conductivity which is caused by the caseinogen, is expressed by the formula

$$\Delta \times 10^5 = 26{\cdot}880 b_1 - \frac{475{\cdot}800}{C} b_1^2 - 2898C.$$

Putting $\Delta = 0$, the concentration of alkali solution b_1 becomes $0{\cdot}000114C$. This proportion of alkali to caseinogen occurs at the point where the change in the conductivity of an alkaline solution, which is brought about by dissolving a given percentage of caseinogen, is zero, and coincides exactly with the value for the solubility of caseinogen which Robertson [1918] and van Slyke and Bosworth [1913] claim to have obtained experimentally.

Van Slyke and Bosworth, however, proved the fact that the caseinogenate of an alkaline earth is not soluble when the proportion of base to caseinogen is $11{\cdot}4 \cdot 10^{-5}$ g. equivalent: 1 g.; it is soluble in water only when the proportion is larger than $22{\cdot}5 \cdot 10^{-5}$. This fact seems to suggest very strongly that we should not identify the solubility of caseinogen in alkali solution with its combining capacity.

In other words, caseinogen may combine with alkali in the proportion of $11{\cdot}4 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen, as deduced from the above-mentioned formula, yet it does not follow that the product of combination must necessarily dissolve in water.

This suspicion with regard to the solubility of van Slyke and Bosworth's monobasic alkali caseinogenate is deepened when we carefully examine the experiments of the authors who claim $11{\cdot}4 \cdot 10^{-5}$ g. equivalent as the solubility as well as the combining capacity of caseinogen to alkali.

When they determined the solubility of caseinogen in alkali, they did not dissolve directly as much caseinogen as possible in alkali solution, but in both investigations a measured amount of caseinogen was dissolved in a known quantity of alkali capable of very easily dissolving the caseinogen taken and then the point was determined at which the first permanent precipitation of caseinogen appeared on neutralising the caseinogen solution with HCl, using the refractometric method (Robertson) or centrifuging (van Slyke and Bosworth). The value, which they call the solubility, is therefore the quantity of alkali used to dissolve the caseinogen minus the amount of alkali neutralised by HCl.

But it must be taken into consideration that the resulting solution of caseinogenate in the experiments of these authors is not a pure aqueous solution of alkali caseinogenate, but a caseinogenate solution containing alkali chloride. Thus the resulting solution in van Slyke and Bosworth's experiment contained almost 0·5 % alkali chloride.

The solubility of caseinogen in NaCl solution seems to be undeniable [Robertson, 1918, p. 88], although it is questioned by van Slyke and Bosworth.

Even if the insolubility of uncombined caseinogen is admitted to be true, as van Slyke and Bosworth claim, yet there remains the possibility that the so-called monobasic caseinogenate may be soluble in alkali chloride solution and insoluble in water, just as in the case of the alkaline earth caseinogenates.

No one has succeeded, so far as I know, in dissolving pure caseinogen (uncontaminated by base) in alkali solution in a proportion of $11{\cdot}5 \cdot 10^{-5}$ g. equivalent directly. Even when we triturate caseinogen in a mortar with

alkali solution for two or three hours, it is not easy to dissolve caseinogen in a proportion of $22{\cdot}5 \cdot 10^{-5}$. It seems hardly credible therefore that the solubility of caseinogen should be more than twice this value. It seems reasonable to ascribe the results obtained by van Slyke and Bosworth as well as by Robertson to the greater solubility of caseinogen in alkali chloride solution or to the solubility of so-called monobasic alkali caseinogenate in alkali chloride solution, if such a combination indeed exists. And the water-soluble combination of alkali and caseinogen should be accepted as containing $22{\cdot}5 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen.

Our experimental results described in the following pages appear to support this view.

Experiment 2. In this experiment, the solubility of caseinogen in pure alkali (NaOH) solution was compared with that of caseinogen in 0·5 % saline solution and that of caseinogen in alkali solution containing 0·5 % alkali chloride (NaCl).

The object of this experiment was to investigate whether the solubility of caseinogen in alkali is increased by the addition of alkali chloride to the alkali solution.

0·4 g. or 0·8 g. of caseinogen was accurately weighed and triturated in a glass mortar respectively with 5 cc. distilled water, 5 cc. 0·5 % NaCl solution, 5 cc. *N*/500 NaOH solution, 5 cc. *N*/500 NaOH solution in 5 % NaCl solution, very thoroughly for about 1·5 hours in each case, and then the mixtures were poured into centrifuge tubes. The mortar was washed with 45 cc. of distilled water in each case and the wash water was also added to the solutions in the tubes. They were then stoppered with cotton wool and were allowed to stand at room temperature (13°–15°) for 24 hours, being shaken from time to time, the air over the solution being changed often so that the CO_2 driven from the solution by the caseinogen was removed.

The solutions were then centrifuged, the supernatant fluid cautiously siphoned off, and the sediment washed carefully with absolute alcohol and ether dried over sodium and then dried and weighed.

By subtracting the obtained weight of sediment from the amount taken (0·4 or 0·8 g.) the amounts of caseinogen dissolved in these four solvents were determined. The results are given in Table IV. The loss of weight of caseinogen triturated with distilled water is apparently manipulative and must be taken into consideration also in the other cases.

Table IV.

Amount of triturated caseinogen. g.	Solvent 50 cc.	Weight of sediment. g.	Amount of caseinogen dissolved. g.
0·4	distilled water	0·3941	0·0059
0·4	0·5 % NaCl	0·3928	0·0072
0·4	*N*/500 NaOH	0·0246	0·3754
0·4	*N*/500 NaOH in 0·5 % NaCl	0·0000	0·4000
0·8	distilled water	0·7922	0·0078
0·8	0·5 % NaCl	0·7885	0·0115
0·8	*N*/500 NaOH	0·4070	0·3930
0·8	*N*/500 NaOH in 0·5 % NaCl	0·0236	0·7764

As will be seen from the table, 50 cc. of NaOH solution, when alkali chloride is added to it, dissolved 0·7764 g. of caseinogen, while the alkali solution alone dissolved only 0·3754–0·3930 g.; that is, the solubility is almost doubled in the former case. That this is not due to the ability of pure NaCl solution to dissolve caseinogen is evident since the amount of caseinogen dissolved in 0·5 % NaCl solution was only 0·0072–0·0115 g.

This experimental result suggests very strongly that the solubility observed by Robertson and Bosworth and by van Slyke is not the solubility of caseinogen in alkali solution but the solubility of caseinogen in alkali solution containing alkali chloride.

Thus it appears very probable that the alkali caseinogenate which contains $11{\cdot}4 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen or the monobasic alkali caseinogenate, as it is called by van Slyke and Bosworth, even if such a substance is admitted to exist, is not soluble in water and soluble only in alkali chloride solution.

As it is impossible to dissolve caseinogen in pure alkali solution in the direct way in larger proportion than $22{\cdot}5$–$25{\cdot}0^{-5}$ g. equivalent alkali per g. caseinogen it seems reasonable to assume that the alkali caseinogenate which is soluble in water must contain this percentage of alkali.

Calculated from this solubility value ($22{\cdot}5$–$25{\cdot}0 \cdot 10^{-5}$), the molecular weight of caseinogen is 4000–4400, one molecule of caseinogen containing one atom of phosphorus and one atom of sulphur.

More evidence must be produced before 8000–8800 can be admitted to be the true molecular weight of caseinogen.

C. The molecular and ionic concentration and the degree of dissociation of alkali caseinogenate of acid reaction.

We have seen in Exp. 1 that the osmotic concentration of alkali caseinogenate solution of neutral and alkaline reactions, is dependent upon the concentration of the alkali in which the caseinogen is dissolved, and that the amount of dissolved caseinogen does not affect in any appreciable degree the osmotic concentration between proportions of alkali to caseinogen from $180 \cdot 10^{-5}$ to $50 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen.

In Exp. 2, the molecular weight of caseinogen was shown to be 4000–4400, if calculated from the solubility of caseinogen in alkali solution. At any rate, the largest ionic weight of the protein radical in the alkali caseinogenate which is soluble in water must be 4000–4400, even if 8800 is ever proved to be the true molecular weight of uncombined caseinogen.

Now, if the alkali bound by caseinogen does not dissociate at all and no inorganic ions are present in the caseinogenate solution, and if polymerisation takes place in a caseinogenate solution of acid reaction while the caseinogen molecule is split successively into smaller protein ions by adding base, as Robertson [1918] has suggested, then the relationship between the osmotic

concentration of alkali caseinogenate solution and the concentration of the alkali in which the caseinogen was dissolved, must be maintained in caseinogenate solutions of acid reaction. Consequently we should expect to be able to obtain 4400 as the molecular weight of caseinogen by means of Barger's method when caseinogen is dissolved in alkali in the proportion of $22{\cdot}5 . 10^{-5}$ g. equivalent alkali per g. caseinogen. But it is quite clear that this expectation cannot be realised experimentally.

4400 will not be obtained by Barger's method for the molecular weight of the caseinogenate at the point of the maximum solubility of caseinogen in alkali solution, simply because the reaction of this caseinogenate solution is acid.

The fact that the reaction of alkali caseinogenate solution is acid when the percentage of base in caseinogenate is smaller than $50 . 10^{-5}$ g. equivalent indicates the presence of free H ions in the solution, that is the molecule of caseinogenate is dissociated into the protein radical and H ions.

The H ion concentration is enhanced from P_H 8·5 to P_H 7·0 when the proportion of alkali to caseinogen is decreased from $80 . 10^{-5}$ to $50 . 10^{-5}$ g. equivalent per g. It will be, therefore, very easily realised that many H ions are dissociated when an alkali solution is saturated with caseinogen. Moreover, the hypothesis that the alkali salt of such a strong acid as caseinogen does not dissociate in the solution at all, is very doubtful. Under these circumstances, we believe that the results obtained in the following experiment may not be very erroneous.

Experiment 3. In this experiment, the molecular and ionic concentration of alkali (NaOH) caseinogenate solution of acid reaction was determined by Barger's method.

Table V.

(*a*) NaOH = *N*/50, caseinogen = 5 %; alkali: caseinogen = $40 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/35–1/40 *M*.
The mean molecular ionic weight = 1875.

(*b*) NaOH = *N*/50, caseinogen = 6 %; alkali: caseinogen = $33{\cdot}3 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/30–1/35 *M*.
The mean molecular ionic weight = 1950.

(*c*) NaOH = *N*/50, caseinogen = 7 %; alkali: caseinogen = $28{\cdot}5 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/30 *M*.
The mean molecular ionic weight = 1750.

Table VI.

(*d*) NaOH = *N*/33·3, caseinogen = 8·0 %; NaOH: caseinogen = $37{\cdot}5 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/20–1/25 *M*.
The mean molecular ionic weight = 1800.

(*e*) NaOH = *N*/33·3, caseinogen = 10·0 %; NaOH: caseinogen = $30 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/17·5–1/20 *M*.
The mean molecular ionic weight = 1875.

(*f*) NaOH = *N*/33·3, caseinogen = 10·8 %; NaOH: caseinogen = $27{\cdot}8 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/17·5–1/20 *M*.
The mean molecular ionic weight = 2025.

(*g*) NaOH = *N*/33·3, caseinogen = 13·5 %; NaOH: caseinogen = $29{\cdot}6 . 10^{-5}$ g. eq.: 1 g.
The molecular and ionic concentration = 1/15–1/17·5 *M*.
The mean molecular ionic weight = 2194.

As shown in Tables V and VI, the osmotic concentration of alkali caseinogenate solution of acid reaction is not dependent upon the concentration of alkali solution in which the caseinogen was dissolved, thus differing from caseinogenate solutions of neutral or alkaline reaction. The factor which conditioned the molecular and ionic concentration of the solution in this experiment was the amount of caseinogen dissolved. The osmotic concentration was almost parallel with the amount of caseinogen dissolved, and consequently the mean weight of the dissolved molecules and ions in the solution was calculated to be about 2000.

The objection might be raised that the result is due to impurities in the caseinogen. But such cannot be the case, because dialysis of the caseinogenate solution did not lower the osmotic concentration of the solution, as shown in the following experiment.

The impurities which may influence the osmotic concentration of the solution would be crystalloid substances having small molecular weights.

Colloids with large molecular weights would not change the osmotic concentration of the caseinogen solution, even though they might happen to be contained in the caseinogen employed. It would, therefore, be expected that dialysis of the caseinogen solution would remove most of any contaminating substance which could influence the osmotic concentration of the solution.

Experiment 4. Caseinogen solution was dialysed in a very thin collodion sack in a cold room for 48 hours, the distilled water outside the collodion tube being changed frequently.

The volume of the solution (0·3 g. caseinogen dissolved in $N/25$ NaOH solution) increased from 3·0 to 8·8 cc.

Table VII.

The molecular and ionic concentration after dialysis = 1/60–1/70 *M*.
The mean molecular ionic weight = 2206.
(The tubes in this experiment stood 32 hours after their preparation.)

Thus in every case, 2000 appears as the mean molecular and ionic weight of alkali caseinogenate when determined by Barger's method, if the proportion of alkali to caseinogen is smaller than $50 \cdot 10^{-5}$ g. equivalent per g. caseinogen.

As has been stated previously, the molecular weight of alkali caseinogenate when determined by calculation from the combining capacity, supposing the solubility of caseinogen in alkali to be $25–22{\cdot}5 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen, must be 4000–4400. We must therefore admit that one molecule of alkali caseinogenate is dissociated into two ions in solutions of acid reaction when the solutions are weak.

In the case of caseinogenate solutions of neutral or alkaline reaction, there may not exist any inorganic ions in the solution as Robertson suggests, the protein molecule being split into protein ions only, but in the case of alkali caseinogenate solutions of acid reaction, it is beyond doubt that the ions in

the solution are mostly those of the protein radical and hydrogen, since the reaction of the solution is acid.

Whether there are any alkali ions dissociated from the caseinogenate molecule, and whether there are any protein ions derived from the splitting of .CO.NH groups, is a very difficult problem to solve.

After I had finished these experiments, my attention was called to the paper of Plimmer and Bayliss [1906] in which they report that caustic soda of strong concentration (1 %) has the power of splitting off P_2O_5 from dissolved caseinogen. Though they admit from their experimental basis that "dilute alkali sufficient only to dissolve the caseinogen causes no separation of soluble P_2O_5," and, as a matter of fact, they themselves employed caustic soda as the solvent of caseinogen in their experiments, and though furthermore the fact that I have obtained quite the same results in my Exp. 1 as those obtained by Robertson and Burnett employing alkaline earths and other alkalies than NaOH, affords clear evidence that the specific decomposing action of caustic soda of strong concentration found by Plimmer and Bayliss was not taking place in my experiments, still I thought it might be of some use to test experimentally whether similar results can be secured when other alkalies than NaOH are used as solvents. Thus I performed a few experiments employing NH_4OH as solvent. The results are as follows.

(I) $NH_4OH = M/50$. Caseinogen = 4 %. Alkali: caseinogen = $50 . 10^{-5}$ g. eq.: 1 g.
Osmotic concentration = 1/47·5 *M*.

(II) $NH_4OH = M/50$. Caseinogen = 6·0 %. Alkali: caseinogen = $33·3 . 10^{-5}$ g. eq.: 1 g.
Osmotic concentration = 1/33·3 *M*.

(III) $NH_4OH = M/33·3$. Caseinogen = 10·8 %. Alkali: caseinogen = $27·8 . 10^{-5}$ g. eq.: 1 g.
Osmotic concentration = 1/15 *M*.

These results are quite the same as observed when NaOH was used as solvent of caseinogen.

Summary.

In the present paper, the solubility of caseinogen and the osmotic concentration of alkali caseinogenate solutions have been investigated, employing Barger's method of determining molecular weights, and it has been found that:

1. The solubility of caseinogen in alkali solution containing a certain amount of alkali chloride, is almost twice that of caseinogen in pure alkali solution.

2. The molecular and ionic concentration of alkali caseinogenate solutions of neutral and alkaline reaction is conditioned by the concentration of the alkali solution in which the caseinogen is dissolved.

3. The osmotic concentration of alkali caseinogenate solutions of acid reaction is dependent upon the amount of dissolved caseinogen, and the mean weight of the ions in the solution is about 2000.

As a result of these experiments, it is suggested that the solubility of caseinogen in alkali solution obtained by Robertson, as well as by van Slyke

and Bosworth, seems very probably to have been that of caseinogen in alkali solution containing alkali chloride. The true solubility of caseinogen in pure alkali, therefore, must be $22{\cdot}5 \cdot 10^{-5}$ g. equivalent alkali per g. caseinogen.

The molecular weight of alkali caseinogenate which is soluble in distilled water must be 4000–4400, and one molecule of caseinogenate seems to be dissociated in such dilute solutions as were investigated (1/15–1/70 *M*) mainly into two ions, consisting principally of H-ions and protein ions, beside the ions of the alkali and split protein.

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REFERENCES.

Plimmer and Bayliss (1906). *J. Physiol.*, **33**, 453.
Robertson (1909). *Univ. of California, Publ. Physiol.*, **3**.
—— (1918). *The Physical Chemistry of Proteins.*
Robertson and Burnett (1909). *J. Biol. Chem.*, **6**, 105.
van Slyke and Bosworth (1913). *J. Biol. Chem.*, **14**, 203, 207, 211.
Yamakami (1920). *Biochem. J.*, **14**, 103.